PATENT COOPERATION TREATY

From the INTERNATIONAL BUREAU

PCT

NOTIFICATION OF ELECTION

(PCT Rule 61.2)

To:

Commissioner
US Department of Commerce
United States Patent and Trademark Office, PCT
2011 South Clark Place Room CP2/5C24
Arlington, VA 22202
ETATS-UNIS D'AMERIQUE

in its capacity as elected Office

Date of mailing (day/month/year) 08 December 2000 (08.12.00)	
International application No. PCT/GB00/01702	**Applicant's or agent's file reference** PPD 50368/WO
International filing date (day/month/year) 04 May 2000 (04.05.00)	**Priority date** (day/month/year) 07 May 1999 (07.05.99)
Applicant SUNER, Marie-Marthe et al	

1. The designated Office is hereby notified of its election made:

   [X] in the demand filed with the International Preliminary Examining Authority on:

   10 October 2000 (10.10.00)

   [ ] in a notice effecting later election filed with the International Bureau on:

   _____

2. The election [X] was

   [ ] was not

   made before the expiration of 19 months from the priority date or, where Rule 32 applies, within the time limit under Rule 32.2(b).

The International Bureau of WIPO
34, chemin des Colombettes
1211 Geneva 20, Switzerland

Facsimile No.: (41-22) 740.14.35

Authorized officer

Olivia TEFY

Telephone No.: (41-22) 338.83.38

GB0001702

PA NT COOPERATION TREAT

From the INTERNATIONAL BUREAU

PCT

NOTIFICATION OF THE RECORDING OF A CHANGE

(PCT Rule 92bis.1 and Administrative Instructions, Section 422)

To:

HUSKISSON, Frank, Mackie
Syngenta Limited
Intellectual Property Dept.
Jealott's Hill Research Station
P.O. Box 3538
Bracknell, Berkshire RG42 6YA
ROYAUME-UNI

Date of mailing (day/month/year)
24 April 2001 (24.04.01)

Applicant's or agent's file reference
PPD 50368/WO

IMPORTANT NOTIFICATION

International application No.
PCT/GB00/01702

International filing date (day/month/year)
04 May 2000 (04.05.00)

1. The following indications appeared on record concerning:

[X] the applicant [ ] the inventor [ ] the agent [ ] the common representative

Name and Address
ZENECA LIMITED
15 Stanhope Gate
London W1Y 6LN
United Kingdom

State of Nationality: GB
State of Residence: GB
Telephone No.:
Facsimile No.:
Teleprinter No.:

2. The International Bureau hereby notifies the applicant that the following change has been recorded concerning:

[ ] the person [X] the name [X] the address [ ] the nationality [ ] the residence

Name and Address
SYNGENTA LIMITED
Fernhurst
Haselmere
Surrey GU27 3JE
United Kingdom

State of Nationality: GB
State of Residence: GB
Telephone No.:
Facsimile No.:
Teleprinter No.:

3. Further observations, if necessary:
This is only a change of name and address. No transfer of patent or other rights has occurred. Agent's address has also been changed accordingly.

4. A copy of this notification has been sent to:

[X] the receiving Office
[ ] the International Searching Authority
[X] the International Preliminary Examining Authority
[ ] the designated Offices concerned
[X] the elected Offices concerned
[ ] other:

The International Bureau of WIPO
34, chemin des Colombettes
1211 Geneva 20, Switzerland

Facsimile No.: (41-22) 740.14.35

Authorized officer
Maria Victoria CORTIELLO

Telephone No.: (41-22) 338.83.38

Form PCT/IB/306 (March 1994)

003979715

PA JT COOPERATION TREAT

From the INTERNATIONAL BUREAU

PCT

NOTIFICATION OF THE RECORDING OF A CHANGE

(PCT Rule 92bis.1 and Administrative Instructions, Section 422)

To:

HUSKISSON, Frank, Mackie
Syngenta Limited
Intellectual Property Dept.
Jealott's Hill Research Station
P.O. Box 3538
Bracknell, Berkshire RG42 6YA
ROYAUME-UNI

Date of mailing (day/month/year)
04 juillet 2001 (04.07.01)

IMPORTANT NOTIFICATION

Applicant's or agent's file reference
PPD 50368/WO

International application No.
PCT/GB00/01702

International filing date (day/month/year)
04 mai 2000 (04.05.00)

1. The following indications appeared on record concerning:

[X] the applicant [ ] the inventor [ ] the agent [ ] the common representative

Name and Address
ZENECA LIMITED
15 Stanhope Gate
London W1Y 6LN
United Kingdom

State of Nationality: GB
State of Residence: GB
Telephone No.:
Facsimile No.:
Teleprinter No.:

2. The International Bureau hereby notifies the applicant that the following change has been recorded concerning:

[ ] the person [X] the name [X] the address [ ] the nationality [ ] the residence

Name and Address
SYNGENTA LIMITED
Fernhurst
Haslemere
Surrey GU27 3JE
United Kingdom

State of Nationality: GB
State of Residence: GB
Telephone No.:
Facsimile No.:
Teleprinter No.:

3. Further observations, if necessary:
UPDATED VERSION

4. A copy of this notification has been sent to:

[X] the receiving Office
[ ] the International Searching Authority
[X] the International Preliminary Examining Authority
[ ] the designated Offices concerned
[X] the elected Offices concerned
[ ] other:

The International Bureau of WIPO
34, chemin des Colombettes
1211 Geneva 20, Switzerland
Facsimile No.: (41-22) 740.14.35

Authorized officer
Maria Victoria CORTIELLO
Telephone No.: (41-22) 338.83.38

PATENT COOPERATION TREATY

PCT

INTERNATIONAL SEARCH REPORT

(PCT Article 18 and Rules 43 and 44)

Applicant's or agent's file reference	**FOR FURTHER ACTION** see Notification of Transmittal of International Search Report (Form PCT/ISA/220) as well as, where applicable, item 5 below.	
International application No.	International filing date *(day/month/year)*	(Earliest) Priority Date *(day/month/year)*
PCT/GB 00/01702	04/05/2000	07/05/1999
Applicant		
ZENECA LIMITED		

This International Search Report has been prepared by this International Searching Authority and is transmitted to the applicant according to Article 18. A copy is being transmitted to the International Bureau.

This International Search Report consists of a total of 3 sheets.

[X] It is also accompanied by a copy of each prior art document cited in this report.

1. **Basis of the report**

a. With regard to the **language,** the international search was carried out on the basis of the international application in the language in which it was filed, unless otherwise indicated under this item.

[ ] the international search was carried out on the basis of a translation of the international application furnished to this Authority (Rule 23.1(b)).

b. With regard to any **nucleotide and/or amino acid sequence** disclosed in the international application, the international search was carried out on the basis of the sequence listing :

[X] contained in the international application in written form.

[X] filed together with the international application in computer readable form.

[ ] furnished subsequently to this Authority in written form.

[ ] furnished subsequently to this Authority in computer readble form.

[ ] the statement that the subsequently furnished written sequence listing does not go beyond the disclosure in the international application as filed has been furnished.

[ ] the statement that the information recorded in computer readable form is identical to the written sequence listing has been furnished

2. [ ] **Certain claims were found unsearchable** (See Box I).

3. [ ] **Unity of invention is lacking** (see Box II).

4. With regard to the **title,**

[ ] the text is approved as submitted by the applicant.

[X] the text has been established by this Authority to read as follows:

UNDIFFERENTIATED ERYTHROID CELLS AND THEIR USE IN LIGAND BINDING ASSAYS

5. With regard to the **abstract,**

[X] the text is approved as submitted by the applicant.

[ ] the text has been established, according to Rule 38.2(b), by this Authority as it appears in Box III. The applicant may, within one month from the date of mailing of this international search report, submit comments to this Authority.

6. The figure of the **drawings** to be published with the abstract is Figure No. _____

[ ] as suggested by the applicant.

[ ] because the applicant failed to suggest a figure.

[ ] because this figure better characterizes the invention.

[ ] None of the figures.

INTERNATIONAL SEARCH REPORT

Interna[redacted] Application No
PCT/GB 00/01702

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 C12N5/16 G01N33/50 C12N15/63

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 C12N G01N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)
EPO-Internal, WPI Data, PAJ, BIOSIS

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	Garcia-Alonso M. et al.:" STABLE FUNCTIONAL EXPRESSION OF THE NEURONAL NICOTINIC ACETYLCHOLINE RECEPTOR alpha3beta4 IN MEL (MURINE ERYTHROLEUKEMIA) CELLS: A NOVEL EXPRESSION SYSTEM FOR LIGAND GATED ION CHANNELS" SOCIETY FOR NEUROSCIENCE ABSTRACTS vol. 22,1996, page 1526 XP000961688 the whole document ---	1-8,19, 22-26
X	GB 2 251 622 A (ICI PLC) 15 July 1992 (1992-07-15) cited in the application figures 13,14 ---	5,7, 18-26
	-/--	

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

° Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
13 November 2000	24/11/2000

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Schönwasser, D

1

Form PCT/ISA/210 (second sheet) (July 1992)

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	NEEDHAM M ET AL: "LCR/MEL: A VERSATILE SYSTEM FOR HIGH-LEVEL EXPRESSION OF HETEROLOGOUS PROTEINS IN ERYTHROID CELLS" NUCLEIC ACIDS RESEARCH, vol. 20, no. 5, 11 March 1992 (1992-03-11), pages 997-1003, XP000602190 ISSN: 0305-1048 cited in the application the whole document	18,20,21
A	WO 98 35020 A (PRESIDENTS AND FELLOWS OF HARV) 13 August 1998 (1998-08-13) figure 1; example 1	13,27-29
A	MIGLIACCIO A.R. ET AL.: "Molecular control of erythroid differentiation" INTERNATIONAL JOURNAL OF HEMATOLOGY, vol. 64, 1996, pages 1-29, XP000926042 the whole document	1-29
A	VANDEN BROECK J. ET AL.: "Characterization of a cloned locust tyramine receptor cDNA by functional expression in permanently transformed Drosophila S2 cells." JOURNAL OF NEUROCHEMISTRY, vol. 64, no. 6, June 1995 (1995-06), pages 2387-2395, XP000926045 cited in the application the whole document	1-29

1

INTERNATIONAL SEARCH REPORT

Information on patent family members

Intern[redacted] Application No
PCT/GB 00/01702

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
GB 2251622 A	15-07-1992	AT 196653 T	15-10-2000
		AU 660636 B	06-07-1995
		AU 9096891 A	22-07-1992
		CA 2058280 A	22-06-1992
		CS 9202671 A	16-12-1992
		DE 69132429 D	02-11-2000
		EP 0516787 A	09-12-1992
		FI 923758 A	20-08-1992
		WO 9211380 A	09-07-1992
		HU 66062 A	28-09-1994
		JP 5504261 T	08-07-1993
		NO 923271 A	16-10-1992
		NZ 241045 A	25-11-1994
		PL 295798 A	12-07-1993
		US 5538885 A	23-07-1996
		ZA 9110015 A	28-10-1992
WO 9835020 A	13-08-1998	EP 1019490 A	19-07-2000

PATENT COOPERATION TREATY

From the
INTERNATIONAL PRELIMINARY EXAMINING AUTHORITY

To:

HUSKISSON, Mackie Frank
ZENECA AGROCHEMICALS
Intellectual Property Department
Jealott's Hill International
Research Centre
PO Box 3538
Bracknell, Berkshire RG42 6YA
GRANDE BRETAGNE

IPD AGROCHEMICALS
ATTORNEY INITIALS DWS
16 AUG 2001
SCANNED
PAGES

PCT

NOTIFICATION OF TRANSMITTAL OF THE INTERNATIONAL PRELIMINARY EXAMINATION REPORT
(PCT Rule 71.1)

Date of mailing *(day/month/year)*	13.08.2001
Applicant's or agent's file reference: PPD 50368/WO	**IMPORTANT NOTIFICATION**

International application No.	International filing date *(day/month/year)*	Priority date *(day/month/year)*
PCT/GB00/01702	04/05/2000	07/05/1999

Applicant: SYNGENTA LIMITED et al.

1. The applicant is hereby notified that this International Preliminary Examining Authority transmits herewith the international preliminary examination report and its annexes, if any, established on the international application.

2. A copy of the report and its annexes, if any, is being transmitted to the International Bureau for communication to all the elected Offices.

3. Where required by any of the elected Offices, the International Bureau will prepare an English translation of the report (but not of any annexes) and will transmit such translation to those Offices.

4. **REMINDER**

   The applicant must enter the national phase before each elected Office by performing certain acts (filing translations and paying national fees) within 30 months from the priority date (or later in some Offices) (Article 39(1)) (see also the reminder sent by the International Bureau with Form PCT/IB/301).

   Where a translation of the international application must be furnished to an elected Office, that translation must contain a translation of any annexes to the international preliminary examination report. It is the applicant's responsibility to prepare and furnish such translation directly to each elected Office concerned.

   For further details on the applicable time limits and requirements of the elected Offices, see Volume II of the PCT Applicant's Guide.

Name and mailing address of the IPEA/

European Patent Office
D-80298 Munich
Tel. +49 89 2399 - 0 Tx: 523656 epmu d
Fax: +49 89 2399 - 4465

Authorized officer

Neumann, M

Tel. +49 89 2399 7351

PATENT COOPERATION TREATY

PCT

INTERNATIONAL PRELIMINARY EXAMINATION REPORT

(PCT Article 36 and Rule 70)

Applicant's or agent's file reference	FOR FURTHER ACTION	See Notification of Transmittal of International Preliminary Examination Report (Form PCT/IPEA/416)
PPD 50368/WO		
International application No.	International filing date *(day/month/year)*	Priority date *(day/month/year)*
PCT/GB00/01702	04/05/2000	07/05/1999
International Patent Classification (IPC) or national classification and IPC		
C12N5/16		
Applicant		
SYNGENTA LIMITED et al.		

1. This international preliminary examination report has been prepared by this International Preliminary Examining Authority and is transmitted to the applicant according to Article 36.

2. This REPORT consists of a total of 6 sheets, including this cover sheet.

   ☐ This report is also accompanied by ANNEXES, i.e. sheets of the description, claims and/or drawings which have been amended and are the basis for this report and/or sheets containing rectifications made before this Authority (see Rule 70.16 and Section 607 of the Administrative Instructions under the PCT).

   These annexes consist of a total of sheets.

3. This report contains indications relating to the following items:

   - I ☒ Basis of the report
   - II ☐ Priority
   - III ☐ Non-establishment of opinion with regard to novelty, inventive step and industrial applicability
   - IV [ ] Lack of unity of invention
   - V ☒ Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations suporting such statement
   - VI [ ] Certain documents cited
   - VII [ ] Certain defects in the international application
   - VIII ☒ Certain observations on the international application

Date of submission of the demand	Date of completion of this report
10/10/2000	13.08.2001
Name and mailing address of the international preliminary examining authority:	Authorized officer
European Patent Office D-80298 Munich Tel. +49 89 2399 - 0 Tx: 523656 epmu d Fax: +49 89 2399 - 4465	Ury, A Telephone No. +49 89 2399 8411

Form PCT/IPEA/409 (cover sheet) (January 1994)

INTERNATIONAL PRELIMINARY EXAMINATION REPORT

International application No. PCT/GB00/01702

I. Basis of the report

1. With regard to the **elements** of the international application *(Replacement sheets which have been furnished to the receiving Office in response to an invitation under Article 14 are referred to in this report as "originally filed" and are not annexed to this report since they do not contain amendments (Rules 70.16 and 70.17))*:
Description, pages:

1-25 as originally filed

Claims, No.:

1-29 as originally filed

Drawings, sheets:

1/10-10/10 as originally filed

2. With regard to the **language**, all the elements marked above were available or furnished to this Authority in the language in which the international application was filed, unless otherwise indicated under this item.

These elements were available or furnished to this Authority in the following language: , which is:

- ☐ the language of a translation furnished for the purposes of the international search (under Rule 23.1(b)).
- ☐ the language of publication of the international application (under Rule 48.3(b)).
- ☐ the language of a translation furnished for the purposes of international preliminary examination (under Rule 55.2 and/or 55.3).

3. With regard to any **nucleotide and/or amino acid sequence** disclosed in the international application, the international preliminary examination was carried out on the basis of the sequence listing:

- ☐ contained in the international application in written form.
- ☐ filed together with the international application in computer readable form.
- ☐ furnished subsequently to this Authority in written form.
- ☐ furnished subsequently to this Authority in computer readable form.
- ☐ The statement that the subsequently furnished written sequence listing does not go beyond the disclosure in the international application as filed has been furnished.
- ☐ The statement that the information recorded in computer readable form is identical to the written sequence listing has been furnished.

4. The amendments have resulted in the cancellation of:

- ☐ the description, pages:
- ☐ the claims, Nos.:

☐ the drawings, sheets:

5. ☐ This report has been established as if (some of) the amendments had not been made, since they have been considered to go beyond the disclosure as filed (Rule 70.2(c)):

(Any replacement sheet containing such amendments must be referred to under item 1 and annexed to this report.)

6. Additional observations, if necessary:

V. Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement

1. Statement

Novelty (N)	Yes:	Claims	1-4, 6, 8-17, 22-29
	No:	Claims	5, 7, 18-21
Inventive step (IS)	Yes:	Claims	1-4, 8-17
	No:	Claims	5-7, 18-29
Industrial applicability (IA)	Yes:	Claims	1-29
	No:	Claims	

2. Citations and explanations
see separate sheet

VIII. Certain observations on the international application

The following observations on the clarity of the claims, description, and drawings or on the question whether the claims are fully supported by the description, are made:
see separate sheet

Item V.

Reference is made to the following documents:

D1: Garcia-Alonso M. et al.:' STABLE FUNCTIONAL EXPRESSION OF THE NEURONAL NICOTINIC ACETYLCHOLINE RECEPTOR alpha3beta4 IN MEL (MURINE ERYTHROLEUKEMIA) CELLS: A NOVEL EXPRESSION SYSTEM FOR LIGAND GATED ION CHANNELS' SOCIETY FOR NEUROSCIENCE ABSTRACTS vol. 22,1996, page 1526 XP000961688

D2: GB-A-2 251 622 (ICI PLC) 15 July 1992 (1992-07-15) cited in the application

D3: NEEDHAM M ET AL: 'LCR/MEL: A VERSATILE SYSTEM FOR HIGH-LEVEL EXPRESSION OF HETEROLOGOUS PROTEINS IN ERYTHROID CELLS' NUCLEIC ACIDS RESEARCH, vol. 20, no. 5, 11 March 1992 (1992-03-11), pages 997-1003, XP000602190 ISSN: 0305-1048 cited in the application

I) Document D1 discloses an erythroid cell (MEL cell) containing and expressing a heterologous protein (the neuronal nicotinic acetylcholine receptor alpha3ß4) under the control of a globin promoter and the LCR (MEL/LCR system). Said MEL cells were induced to differentiate with DMSO.
D1 further discloses that said MEL/LCR system affords advantages for the detection of functional activity of receptors and ion channels.
In other words, D1 discloses or at least suggests the method according to present claims 18-21 (Article 33.2 and 3 PCT).

II) The difference between the vectors disclosed in D3 (page 998) and the vector according to present claims 22-25 is that the latest comprises a sequence which encodes a non-mammalian protein receptor.
The difference between the vector disclosed in D1 (second part of the document) and the vector according to present claims 22-25 is that the latest comprises a sequence which encodes a protein receptor which is non-mammalian.
In view of the advantages of the MEL/LCR system for reproducible, high-level expression and stable expression of heterologous proteins of interest (see D1 and D3), the skilled person would have used said system for the production of e.g. non-mammalian protein receptor. The substitution of the proteins according to D1

and D3 with a non-mammalian protein receptor in the MEL/LCR system merely consists in an obvious alternative which does not involve an inventive step. Therefore, the vector (intermediate product) according to claims 22-25 does not fulfil the requirements of Article 33.3 PCT.

The undifferentiated erythroid cell according to claim 26 is an intermediate cell obtained in the MEL/LCR system before induction of differenciation has started. Said cell is also not inventive since it is compulsorily obtained in the procedure.

III) The undifferentiated erythroid cell according to claim 5 is an intermediate cell obtained in the MEL/LCR system disclosed in D1 and D3 before induction of differenciation has started. Thus, claim 5 lacks novelty (Article 33.2 PCT). The method according to claim 7 is also disclosed in D3 (pages 998-999).

Dependent claims 6, 27-29 do not seem to contain any feature which, in combination with the features of the claims to which they refer, meet the requirements of the PCT in respect of inventive step.

IV) D2, figs.13 and 14 (see NI = non-induced) also destroy the novelty of present claims 5 and 6.

V) In the conventional MEL/LCR system, induction of differentiation frequently leads to the loss of functionality of the signalling cascades linked to G-protein coupled receptors. Thus recombinant MEL cell cannot be used in functional assays with G-protein coupled receptors. The present invention is based on the fact that undifferentiated erythroid cells (MEL cells for instance) can be used in functional assays with G-protein coupled receptors (Ca++, IP3, or cAMP assays) since the signalling cascades linked to G-protein coupled receptors are still functional. This was neither disclosed nor suggested in the cited prior art.
Thus, it would seem that the subject-matter of claims 1-4 and 8-17 fulfil the requirements of Article 33.2 and 3 PCT.

Item VIII.

1) It is clear from the description that the "heterologous protein" (claim 1) and the "receptor protein" (claim 8) should be defined as being a "G-protein coupled receptor". This feature is essential to the definition of the invention.
Since independent claims 1 and 8 do not contain this feature they do not meet the requirement following from Article 6 PCT taken in combination with Rule 6.3(a) PCT that any independent claim must contain all the technical features essential to the definition of the invention.

2) The term "substantially" used in claims 1, 5 is vague and unclear and leaves the reader in doubt as to the meaning of the technical features to which it refers, thereby rendering the definition of the subject-matter of said claims unclear (Article 6 PCT).

3) The expressions "thereof" (claims 1, 5) and "a cell as defined above" (claim 8) are unclear (Article 6 PCT).

(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
16 November 2000 (16.11.2000)

PCT

(10) International Publication Number
WO 00/68362 A3

(51) International Patent Classification[7]: C12N 5/16, G01N 33/50, C12N 15/63

(21) International Application Number: PCT/GB00/01702

(22) International Filing Date: 4 May 2000 (04.05.2000)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
9910664.3 7 May 1999 (07.05.1999) GB

(71) Applicant *(for all designated States except US)*: **ZENECA LIMITED** [GB/GB]; 15 Stanhope Gate, London W1Y 6LN (GB).

(72) Inventors; and

(75) Inventors/Applicants *(for US only)*: **SUNER, Marie-Marthe** [FR/GB]; Jealott's Hill International Research Centre, Bracknell, Berkshire RG42 6ET (GB). **WINDASS, John, David** [GB/GB]; Jealott's Hill International Research Centre, Bracknell, Berkshire RG42 6ET (GB). **EARLEY, Fergus, Gerard, Paul** [IE/GB]; Jealott's Hill International Research Centre, Bracknell, Berkshire RG42 6ET (GB). **DUNBAR, Stuart, John** [GB/GB]; Jealott's Hill International Research Centre, Bracknell, Berkshire RG42 6ET (GB). **BLYTHE, Judith, Lesley** [GB/GB]; Jealott's Hill International Research Centre, Bracknell, Berkshire RG42 6ET (GB).

(74) Agents: **HUSKISSON, Frank, Mackie** et al.; Zeneca Agrochemicals, Intellectual Property Dept., Jealott's Hill Research Station, P.O. Box 3538, Bracknell, Berkshire RG42 6YA (GB).

(81) Designated States *(national)*: AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CR, CU, CZ, DE, DK, DM, DZ, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, TZ, UA, UG, US, UZ, VN, YU, ZA, ZW.

(84) Designated States *(regional)*: ARIPO patent (GH, GM, KE, LS, MW, SD, SL, SZ, TZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).

Published:
— *With international search report.*

(88) Date of publication of the international search report:
15 March 2001

For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

WO 00/68362 A3

(54) Title: UNDIFFERENTIATED ERYTHROID CELLS AND THEIR USE IN LIGAND BINDING ASSAYS

(57) Abstract: The use of an erythroid cell which is substantially undifferentiated but which is capable of expressing a heterologous protein under the control of a globin promoter thereof, in an assay in which said protein interacts with an endogenous signalling cascade of said cell and said interaction is detected. In general, such assays will be functional assays. The production of suitable erythroid cells as well as cells useful in this way are also described and claimed.

INTERNATIONAL SEARCH REPORT

Inte. onal Application No
PCT/GB 00/01702

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 C12N5/16 G01N33/50 C12N15/63

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 C12N G01N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)
EPO-Internal, WPI Data, PAJ, BIOSIS

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	Garcia-Alonso M. et al.:" STABLE FUNCTIONAL EXPRESSION OF THE NEURONAL NICOTINIC ACETYLCHOLINE RECEPTOR alpha3beta4 IN MEL (MURINE ERYTHROLEUKEMIA) CELLS: A NOVEL EXPRESSION SYSTEM FOR LIGAND GATED ION CHANNELS" SOCIETY FOR NEUROSCIENCE ABSTRACTS vol. 22,1996, page 1526 XP000961688 the whole document ---	1-8,19, 22-26
X	GB 2 251 622 A (ICI PLC) 15 July 1992 (1992-07-15) cited in the application figures 13,14 ---	5,7, 18-26
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[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

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Date of the actual completion of the international search	Date of mailing of the international search report
13 November 2000	24/11/2000

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl Fax (+31-70) 340-3016	Schönwasser, D

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INTERNATIONAL SEARCH REPORT

Inte. onal Application No
PCT/GB 00/01702

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication,where appropriate, of the relevant passages	Relevant to claim No.
X	NEEDHAM M ET AL: "LCR/MEL: A VERSATILE SYSTEM FOR HIGH-LEVEL EXPRESSION OF HETEROLOGOUS PROTEINS IN ERYTHROID CELLS" NUCLEIC ACIDS RESEARCH, vol. 20, no. 5, 11 March 1992 (1992-03-11), pages 997-1003, XP000602190 ISSN: 0305-1048 cited in the application the whole document	18,20,21
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A	MIGLIACCIO A.R. ET AL.: "Molecular control of erythroid differentiation" INTERNATIONAL JOURNAL OF HEMATOLOGY, vol. 64, 1996, pages 1-29, XP000926042 the whole document	1-29
A	VANDEN BROECK J. ET AL.: "Characterization of a cloned locust tyramine receptor cDNA by functional expression in permanently transformed Drosophila S2 cells." JOURNAL OF NEUROCHEMISTRY, vol. 64, no. 6, June 1995 (1995-06), pages 2387-2395, XP000926045 cited in the application the whole document	1-29

1

INTERNATIONAL SEARCH REPORT

Information on patent family members

Inte .onal Application No
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Patent document cited in search report	Publication date	Patent family member(s)	Publication date
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		AU 660636 B	06-07-1995
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(54) Title: CELLS AND ASSAYS

(57) Abstract

The use of an erythroid cell which is substantially undifferentiated but which is capable of expressing a heterologous protein under the control of a globin promoter thereof, in an assay in which said protein interacts with an endogenous signalling cascade of said cell and said interaction is detected. In general, such assays will be functional assays. The production of suitable erythroid cells as well as cells useful in this way are also described and claimed.

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Inventors; and

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CELLS AND ASSAYS

The present invention relates to cells and particularly erythroid cells, to methods of producing them and their use in assays. as well as to vectors useful in the methods.

In animal cell expression, the ideal has always been to have a system capable of reproducible, high level, stable expression of the proteins of interest. The LCR/MEL expression system (Locus Control Region/Murine Erythroleukemia cells) complies with this demand as previously demonstrated by Amar *et al.*, (1995) J. Recept. Signal Tr. R. **15** 71-79; Egerton *et al.*, (1995) J. Mol. Endocrinol. **14** 179-189; Needham *et al.*, (1992) Nucleic Acids Res. **20** 997-1003, Needham *et al.*, (1995) Protein Expres. Purif. **6** 124-131; Newton et al., (1994) Protein Expres. Purif. **5** 449-457 and Shelton *et al.*, (1993) Receptor. Channel. **1** 25-37.

MEL cells are erythroid progenitor, robust, semi-adherent cells with a doubling time of only 10 to 16 hours, which are derived from spleens of susceptible mice infected with the Friend Virus Complex (Friend C (1957) J. Exp. Med. **105** 307-318). They are continuously dividing cells, arrested at the proerythroblast stage. Changes similar to normal red blood cell maturation can be induced with a variety of chemical agents, including polar-planar compounds like dimethyl sulfoxide (DMSO). This terminal differentiation causes an increase of globin gene expression which can result in α and β globin comprising up to 25 % of the total cell protein. The globin LCR enhancer is responsible for high levels of erythroid cell specific expression of globin proteins.

The human globin LCR has been utilised in the LCR/MEL system, alongside a human β-globin promoter *in cis*, to drive integration site position independent expression of cDNA and genomic sequences (Needham *et al.*, 1995 supra.). The globin LCR confers integration site independent expression on stably transfected genes which are linked *in cis* (Blom von Assendelft et al. (1989) Cell **56** 969-977; Talbot et al. (1989) Nature **338** 352-355). The human β-globin promoter and parts of the β-globin gene provide mRNA processing and maturation signals, give stability to the final mRNA and confer high expression levels in induced cells (Needham *et al.*, 1992 supra).

The LCR/MEL system has already been used to express a variety of proteins. Stable expression of electrophysiologically functional mammalian homo- and hetero-multimeric ion channel proteins has been obtained in MEL cells (Amar *et al.*, 1995 supra.; Shelton *et al.*,

1993 supra. and Monica Garcia-Alonso (1997) "Evaluation of the potential of Murine Erythroleukemia (MEL) cells as an expression system for nicotinic acetylcholine receptors" (Ph.D. thesis Reading University UK)). The LCR/MEL system is capable of producing functional secreted proteins (Needham *et al.*, 1992 supra.; Newton *et al.*, 1994 supra.). It has also been shown to produce very high levels of mammalian G-protein coupled receptors (sometimes known as seven-transmembrane helix receptors or 7TMR) as a source for ligand binding experiments (Egerton *et al.*, 1995 supra.; Needham *et al.*, 1995 supra.).

UK Patent No. 2251622 describes and claims expressions systems, including those based upon MEL cells, for the expression of heterologous polypeptides, in particular human proteins such as human growth hormone.

All of the previous examples of expression from MEL cells were of proteins of mammalian origin. High level expression of the genes was only seen after the recombinant MEL cell differentiation had been induced, although some background expression of a heterologous reporter gene (CAT) under the influence of an α-globin promoter in uninduced MEL cells has been described (Pondel et al., Nucl. Acids Res. 20, 2, 237-243).

There are several approaches to achieve stable, heterologous expression of G protein-coupled receptors in animal cells (Vanden Broeck, 1996, Int. Rev. Cytol. 164, 189-268). The majority of examples have come from mammalian systems. In conventional mammalian cloning systems, it can prove to be labour intensive to produce stable recombinant cell lines reliably expressing large amounts of receptor as well as to produce large numbers of recombinant cells.

The LCR/MEL cell expression system resolves these problems, as it is capable of reproducible, high level, stable expression of receptors as well as being a robust semi-adherent cell line (Needham *et al.*, 1992). However, with the conventional LCR/MEL system, heterologous protein expression only occurs at high levels after induction of differentiation of the cells into mature red blood cells. Unfortunately, this differentiation frequently leads to the loss of functionality of the signalling cascades usually linked to G protein-coupled receptors. This means that the existing LCR/MEL system is ideal for ligand binding assays on recombinant MEL cells, but cannot be used in functional assays (Ca^{2+}, IP3 or cAMP assays). As a consequence, functional assays with G-protein coupled receptors have to be performed in other systems.

According to the present invention there is provided the use of an erythroid cell which is substantially undifferentiated but which is capable of expressing a heterologous protein under the control of a globin promoter thereof, in an assay in which said protein interacts with an endogenous signalling cascade of said cell and said interaction is detected.

Such assays are functional assays. Ligand binding assays may also be effected if the cells are induced prior to assay.

Suitable erythroid cells are murine erythroleukaemia (MEL) cells, rat erythroleukaemia cells (REL) and human erythroleukaemia cells (HEL), but are preferably murine erythroleukaemia cells.

Particular globin promoters which control expression of proteins in the cells of the invention are the β-globin promoters, such as human β-globin promoters.

In a further aspect, the invention provides an erythoid cell which is substantially undifferentiated but which is capable of expressing proteins under the control of a globin promoter thereof at levels which allow use as described above.

Cells in accordance with the invention can be obtained by culture of uninduced erythroid cells for a sufficient period of time, usually over a period of a few months, until they become "leaky" in the sense that protein under the control of globin promoters are expressed.

Leakiness in cells can be detected by routine methods. For example, the cells can be screened for mRNA levels using for example Northern blotting techniques. Detection of protein mRNA, for example β-globin mRNA would be sufficient to indicate that the cells were in the correct stage.

Alternatively, the cells can be transformed with a reporter or marker gene which is placed under the control of a globin promoter, preferably a β-globin promoter, and detecting expression of the marker gene in uninduced cells. Suitable reporter or marker genes for use in this process are well known in the art and include for instance the β-galactosidase gene (β-Gal).

In a further alternative, it may be possible to determine that the cells are in the correct state because they are beginning to change colour, by taking on a pinkish hue, indicating that a progression to red differentiated erythroid cells has begun. This may be detected either spectrometrically or by eye.

A yet further alternative is to detect expressed proteins themselves, for example using a conventional antibody type assay which may be either a direct or competitive assay. Examples of suitable proteins which may be detected include globins. Means of carrying out such assays are well known in the literature and include the use of labelled antibodies, for example radiolabelled or fluorescent antibodies, as well as enzyme-linked immunoassays (ELISAs).

A particular type of cell which can form cells of the invention are subclones of the MEL C-88 cell line, an example of which was deposited at the European Collection of Cell Cultures under the Accession number 99012801, deposited on 28 January 1999. This clone has been designated "MEL-C88L".

Cell lines of this type can be used in functional assays as illustrated hereinafter, since the cells retain nucleii which are lost or otherwise functionally silenced on terminal erythroid differentiation. Thus use can be made of the signalling pathways in the cell, such as those in which G-proteins are involved, where for example, globin promoters can drive the expression of heterologous proteins which normally functionally interact with a G-protein, in particular G-protein coupled receptor molecules (GPCR). These receptor molecules may be of mammalian or non-mammalian origin and in particular are insect receptors such as the *Locusta migratoria* tyramine receptor (TyrLoc), or other receptors such as dopamine, octopamine, serotonin, or acetylcholine receptors such as muscarinic acetylcholine receptors.

In a further aspect, the invention provides a method for determining the interaction between a receptor protein and a potential agonist or antagonist therefor, said method comprising incubating a cell as defined above which has been transformed so that it expresses said receptor protein as a G-protein coupled receptor,

either (I) in (a) the presence and (b) the absence of said potential agonist; and/or

(II) in the presence of a known agonist and (a) the presence or (b) the absence of said potential antagonist; and measuring and comparing G-protein induced signals in cells of (Ia) and (Ib) and/or (IIa) and (IIb).

The G-protein coupled receptor signal is induced in the presence of ligands for that receptor. In such a case, the G-protein coupled receptor which is expressed in MEL C88L cells, interacts with the G-protein and triggers a signalling cascade which may either increase or decrease the concentrations of various detectable components within the cell. Mechanisms by which these signalling cascades may operate are illustrated in the literature,

for example, S. Klostermann et al., Perspectives in Neurobiology, (1996) 4, 237-252 and M.A.D. Fazia, FEBS Letters 410 (1997) 22-24.

The levels of these signals may be indicative of agonist or antagonist activity. This is particularly applicable if other possible target sites in the signalling cascade on which the compound may act have been eliminated, for example by carrying out specific assays for the other possible target sites, carrying out assays on untransformed cells, and/or using electrophysiological assays or studies on receptors or receptor preparations. Whether these changes are the result of the presence of the specific candidate agonist or antagonist can then be established by observing the difference between the signals generated by the cell line expressing the receptor protein of interest and the untransformed progenitor cell line i.e. cell lines differing only in the presence/absence of the target receptor. Where an elevated or heightened G-protein coupled receptor induced signal results in an elevation of the amounts of a particular downstream component in the signalling cascade, the signal generated by (Ia) will be greater than that generated by (Ib) if the potential agonist is effective. Under similar circumstances, the signal from step IIa above will be lower than that obtained in IIb if the potential antagonist is effective. Conversely, if an elevated G-protein coupled receptor induced signal results in a decrease in the concentration of a particular downstream component in the signalling cascade, application of a potential agonist would result in a reduction of the levels of that component. This can often only be detected by amplifying the signal using a chemical which artificially stimulates the level of the component e.g. forskolin. Sometimes, a further chemical which artificially inhibits an enzyme in the pathway is also added, as this again contributes to an increase in the level of the component. An example of such a further chemical is 3-isobutyl-1-methylxanthine (IBMX). Step (Ia) will then be lower than step (Ib). Where an antagonist in being assayed, the results of step (IIa) will be greater than (IIb), but can only be detected in the presence of forskolin.

In one embodiment, the G-protein coupled receptor induced signal is monitored by measuring the free calcium ion concentration of the cells. This can be done using known techniques, for example utilising a fluorescent indicator, such as fura-2 which binds free calcium ions, and whose fluorescent signal alters depending upon whether it is bound to calcium ions or free.

In an alternative embodiment, the G-protein coupled receptor induced signal is monitored by measuring the cyclicAMP (cAMP) levels within the cell, which may be

increased or decreased, depending upon the nature of the G-protein coupled receptor. The G-protein coupled receptor induced signal activates the G-protein which interacts with adenylate cyclase enzyme either to increase or decrease the levels of cAMP in the cell. cAMP can be extracted from the cells and quantified using commercially available kits such as scintillation proximity assay (SPA) kits available from Amersham International (UK).

The calcium signalling mechanism is similar in that the concentration of calcium ions found in cells changes (either increases or decreases) as a result of G-protein induced signal.

In yet a further embodiment, the cells may be further transformed with a reporter or marker gene, such as β-galactosidase, expression of which is regulated by the G-protein coupled receptor signalling cascade. Changes in the signal will then be apparent by detecting the expression levels of the marker gene.

If necessary, where the G-protein coupled receptor induced signal results in a decrease in the level of the measured cellular component, the changes in the signal can be amplified by adding chemicals which either stimulate or inhibit components in the signalling pathway so resulting in an increase of the detectable chemical in the cell. For example, forskolin (FSK) is known to artificially stimulate cAMP levels in a cell by directly activating adenylate cyclase. 3-Isobutyl-1-methylxanthine (IBMX) is known to artificially increase cAMP levels in the cell by inhibiting cAMP phosphodiesterase. Hence when the G-protein coupled receptor signal results in a decrease in cAMP levels, this decrease may appear more clearly if the agonist, with or without antagonist, is added in the presence of forskolin and optionally also IBMX. An effective agonist would be expected to reduce the amount of cAMP as compared to the forskolin, or the forskolin and IBMX alone. The presence of an effective antagonist would mean that the levels of cAMP would be higher than the test (IIb), in the absence of the antagonist.

In such cases, a further assay, in the presence of forskolin, forskolin and IBMX or other artificial stimulants alone may be of assistance in the determining the levels of efficacy of the agonist or antagonist, and or in assessing the relative efficacies of various potential agonists and antagonists.

In all cases, cells of the invention are first transformed so that they express the G-protein coupled receptor of interest and clones which provide a good G-protein coupled receptor induced signal can be selected by testing each clone by adding varying amounts of the known receptor ligand, and if necessary, a chemical which stimulates the detectable

cellular component (as discussed above). Clones which provide the most clearly distinguishable signals as between (Ia) and (Ib) and/or (IIa) and (IIb) above are selected for use in assays.

Cells of the invention can be used to express heterologous proteins, including human proteins. In particular however, the applicants have found that they are useful in the expression of non-mammalian and especially insect proteins.

To date, MEL cells giving a significant background level of G-protein coupled receptor expression in the absence of inducers such as dimethylsulphoxide (DMSO), have been used in a number of independent transformation experiments. In each case, even where a small number of transformed clones, for example ≥3, were analysed, isolates showing both efficient inducible but low level "leaky" expression of the introduced heterologous genes were identified.

The applicants have, for the first time, expressed an insect G-protein coupled receptor in the LCR/MEL system: the locust tyramine receptor, TyrLoc. This receptor was previously expressed in stably transfected *Drosophila* S2 cells (Vanden Broeck et al. (1995) J. Neurochem. **64** 2387-2395). The present results with the MEL-TyrLoc cells indicate that the pharmacology of the receptor expressed in these mammalian cells is similar to that in S2-TyrLoc cells. Both Ca^{2+} and cAMP measurements demonstrate that there is a very efficient coupling of the expressed insect receptor to the endogenous, mammalian G proteins. This observation indicates that the use of this novel MEL expression system should not necessarily be restricted to the characterization and functional analysis of mammalian receptor proteins. Moreover, the ability of the LCR/β-globin promoter combination to confer high levels of expression, in a reproducible and position independent manner, is not affected in this MEL C88L cell clone.

This was shown by the fact that the monophenolic amine, tyramine (TA) is a much better agonist than octopamine (OA). It activates this receptor at concentrations which are 3-4 orders of magnitude lower than OA. Also, yohimbine proved to be a better receptor antagonist than chlorpromazine and mianserin.

These results clearly confirm the ligand binding data and the attenuating effect of TA on forskolin stimulated cAMP production which were previously obtained with Tyr*Loc* expressing S2 cells (Vanden Broeck *et al.*, 1995 supra.). G protein-coupled receptors for phenolamines (TA and/or OA) have been identified in other insect species (*Drosophila*

melanogaster: Arakawa et al. (1990) Neuron **2** 343-354 and Saudou et al., (1990) EMBO J. **9** 3611-3617; *Heliothis virescens*: Von Nickisch-Rosenegk et al., (1996) Insect Biochem. Molec. Biol. **26** 817-827) and in the mollusc *Lymnea stagnalis* (Gerhardt CC et al., (1997) Mol. Pharmacol. **51** 293-300). The *H. virescens* (K50*Hel*) and *L. stagnalis* (*Lym*-OA1) receptors are preferentially activated by OA, whereas the fruitfly receptor (Tyr/Oct-*Dro*) produces agonist-specific (TA versus OA) coupling to different second messenger systems when it is expressed in NIH 3T3 (Saudou *et al.*, 1990 supra.), in CHO cells (Robb (1994) EMBO J. **13** 1325-1330) or in *Xenopus* oocytes (Reale et al. (1997) Brain Res. **769** 309-320).

Assays using the cells of the invention and methods in accordance with the invention have been shown to be effective in determining the relative strengths of receptor agonists and antagonists. Specifically, it has been found that the locust receptor Tyr*Loc* is more sensitive to TA than OA as agonists for both cAMP inhibition and Ca^{2+} stimulation in MEL-TyrLoc cells.

These assays are useful in investigations into the biological function of molecules. For example, it is known that TA is the biosynthetic precursor of OA and it is present in many parts of the locust nervous system. The results reported here also imply that TA might be a very important neuro-active substance and this idea is strongly supported by the discovery of separate activities, binding sites and uptake systems for tyramine and octopamine in the locust central nervous system (Roeder T (1994) Comp. Biochem. Physiol. **107C** 1-12; Hiripi L et al., (1994) Brain Res. **633** 119-126.,; Downer et al., (1993) Neurochem. Res. **18** 1245-1248).

After induction, this clone also differentiates along the erythroid pathway and as a result boosts the expression levels of heterologous TyrLoc receptor proteins at least three- to four-fold. As a consequence, when induced, this clone loses the functionality of its signal transduction pathway, but is now ideal for ligand binding assays.

Thus the invention further provides an assay for detecting binding between a protein and a potential binding partner therefore, said method comprising (a) transforming a cell as described above so that the protein is expressed under the control of a globin promoter, (b) detecting binding between said potential binding partner and the protein on the membrane of the cell. Optionally, the cells are induced after step (a) and prior to step (b), so as to obtain high levels of protein expression from fully differentiated cells.

Step (b) may be effected on whole cells, or on isolated membranes extracted from lysed cells. Suitably, the protein is a receptor and the potential binding partner is a ligand therefore. However, binding between other types of protein, such as naturally occuring proteins, antigens, immunoglobulins such as antibodies, and binding partners, in particular specific binding partners can be tested in this manner.

This work demonstrates that the MEL cell line can be an even more versatile system than previously thought. It may be used in a variety of situations, from functional (G-protein coupled receptor signalling cascade or ligand gated ion channels) to ligand binding assays, for both mammalian and insect, secreted or transmembrane proteins.

Vectors used in tranformation of the cell lines form a further aspect of the invention.

Preferably, a "parent" cell line is established for use in the assays described above. This is a cell line comprising cells of the invention which have been transformed such that they express a suitable reporter gene such as the LacZ gene, under the control of a response element which is susceptible to modulation by a signalling cascade used in the assay. An example of such a response element would be a cAMP response element (CRE). The reporter gene is suitably also under the control of a minimal promoter, for example a minimal globin promoter. Suitable enhancers may also be included in order to ensure good expression levels. A particular enhancer is the LCR enhancer described above. Suitably, the distance and/or orientation between the enhancer and the promoter is arranged such that good or optimal induction of expression of the reporter gene by an increase in the concentration of a particular downstream component in the signalling cascade (such as cAMP mentioned above) is ensured.

The parent cell line may additionally comprise a globin promoter and preferably also an enhancer, in particular LCR, arranged to enhance expression of a gene placed under the control of the globin promoter. A multiple cloning site is suitably provided adjacent the globin promoter such that various protein genes, and in particular receptor genes, may be introduced into the parent line as required.

Suitable parent cell lines may be obtained using empirical methods, for example by co-transforming cells with a vector including the reporter gene under the control of a response element and, for instance, a minimal globin promoter, and subjecting the cells to the target assay conditions and selected clones which produce the best signal (see Examples 7 and 8 hereinafter). An example of such a co-transformation system is the reporter cassette in

the p3XVIP hyg (P) vector illustrated hereinafter, and a vector containing the LCR/globin gene promoter such as the pEV3 vector illustrated hereinafter.

In order to determine which clones have stably incorporated both vectors, each vector should contain a different selection marker gene, such as antibiotic resistance. For example, the p3XVIP hyg (P) vector includes a hygromycin resistance gene which confers resistance to hygromycin B, whilst the pEV3 vector includes a neomycin resistance gene which confers resistance to G418. The presence of this neomycin resistance gene in the parent cells hinders the transformation of this cell line with a vector allowing expression of an heterologous protein, when this protein is encoded by a gene cloned into a similar parent pEV3 vector.

Thus suitably other selection genes/chemical systems are utilised , e.g. a mammalian promoter, like the TK promoter in pEV3 (Figure 1a), driving the expression of a Blasticidin-S deaminase enzyme (e.g. from the bsd gene of *Aspergillus terreus*), which is responsible for resistance to Blasticidin S.

Another way of obtaining such a parent cell line would be, for example to investigate the optimal organisation (distance, orientation etc.) of, say the LCR enhancer, such as the one in the pEV3 vector, and the reporter cassette, such as is carried by the p3XIP hyg (P) vector (and for this, the optimal clones selected using the above method would be useful) and reproduce this organisation in a single vector. This vector could then be utilised to assemble a parent cell line in MEL C88L or to co-transfect it with the expression vector. In yet a further alternative, the optimal organisation of enhancer/response element/minimal promoter/ reporter gene can be identified (e.g. LCR/ CRE (3xVIP) - β globin minimal promoter - LacZ) and this could then be reproduced into a pEV3 like vector to provide both expression and reporter cassettes in the same vector.

The selection marker gene such as bsd as mentioned above, could either be incorporated into a single vector used to assemble the parent cell line, or it could be included in a vector used in co-transfection, such as new derivative of the pEV3 vector, pEV3/Blasto, where the neomycin resistance cassette is removed. The vector pEV3/Blasto could subsequently be utilised for expression of heterologous proteins affecting the cAMP signalling pathway, in a previously generated parent cell line where the expression of the reporter gene depends on an increase on the concentration of cAMP.

Use of a pre-characterised parent cell line is advantageous in that it harnesses the full benefits of using the LCR element to reduce numbers of clones which must be screened

before "good expressers" are obtained. It would ensure that a clone with good inducibility of β–galactosidase expression as well as the capacity to express high levels of heterologous protein is obtained, even when only small numbers of clones (e.g. 6 clones/GPCR expression system) are characterised.

In the absence of a parent cell line of this type, generation of a cell line providing good expression levels of the GPCR receptor and a robustly and usefully inducible β–galactosidase reporter gene necessitates construction and analysis of a minimum of 25 to 50 clones because the reporter element is not itself associated with an LCR element. These numbers of clones are required to provide a reasonable chance of obtaining a clone that expresses a heterologous receptor at a suitable, together with the necessary inducibility of β–galactosidase expression.

In turn, the use of a parent cell line should also allow easier parallel assembly of cell lines expressing different GPCRs and/or different expression modules for the same GPCR. Higher productivity in useful cell line assembly can therefore be achieved.

A further major advantage of such a parent cell line is one of consistency: without it, from one transformation to the next it is impossible to obtain the same levels of inducibility of β–galactosidase expression because of more-or-less subtle position effects. A parent cell line avoids any difficulties in isolating a clone were the induction is robust enough to allow use of the cell line in high throughput screening assays. In turn, this reduces the amount of time necessary for characterising the clones obtained from a transformation, as well as the time necessary to develop a robust assay for high throughput screening. Consequently, by using a parent cell line the number of clones isolated from each transfection can be minimised, the consistency of β–galactosidase induction levels increased and the costs reduced.

Parent cell lines of this type form a further aspect of the invention.

The invention will now be particularly described by way of example with reference to the accompanying diagrammatic drawings in which:

Figure 1a illustrates the expression vector pEV3 used as a recipient for the locust tyramine receptor (TyrLoc), such that the globin LCR enhancer and the murine β-globin promoter drive integration site independent expression of the receptor cDNA;

Figure 1b illustates a reporter vector p3XVIP-hyg(P) which comprises three copies of a cAMP response element (3XVIP) upstream a minimal β-globin promoter arranged to drive the expression of a β-galactosidase reporter gene in the presence of cAMP in the cell;

Figure 2 is a graph showing the dose-response relationship between tyramine (TA) concentration and Ca^{2+} increase in MEL-TyrLoc cells compared to MEL C88L control cells which do not express the receptor;

Figure 3 is a graph showing a comparison of calcium responses in MEL-TyrLoc cells elicited by tyramine (TA) (10 nM) and octopamine (OA) (0.5 μM);

Figure 4 is a graph illustrating that the addition of different concentrations of yohimbin (Y) results in an inhibition of the response to 0.5 μM TA, but could not inhibit the response to high TA concentrations (5 μM);

Figure 5 is a graph showing control cAMP measurements of untransformed MEL-C88L cells in the presence of forskolin (FSK);

Figure 6 illustrates the inhibition of forskolin induced increase of cAMP by tyramine in MEL-TyrLoc cells;

Figure 7 illustrates the results obtained with a β-gal assay using MEL cells transformed with a dopamine receptor and the reporter vector where, in each group, the first column indicates cells incubated in medium alone, the second shows those incubated with 3μM forskolin, the third column shows the results of incubation with 10μM dopamine;

Figure 8a illustrates the results obtained with a β-gal assay using MEL C88L cells transformed with the reporter vector p3XVIP hyg (P) where, in each group, the first column indicates cells incubated with 3.6μM forskolin and the second shows those incubated in medium alone; and Figure 8b illustrates the results obtained with a β-gal assay using MEL C88L cells co-transformed with the reporter vector p3XVIP hyg (P) and the expression vector pEV3 where, in each group, the first column indicates cells incubated with 3.6μM forskolin and the second shows those incubated in medium alone.

Example 1

Generation of the pEV3TyrLoc expression vector

The tyramine receptor cDNA from the locust *Locusta migratoria* was cloned into pEV3, downstream of the human globin locus control region (LCR) between the promoter and the second intron of the β-globin gene (Figure 1a) as follows.

The coding region of the TyrLoc cDNA was amplified by polymerase chain reaction (PCR) from pVJ12 and pVJ12-IEG (J. Vanden Broek et al., J. Neurochemistry (1995) 64, 6, 2387-2395) using the following oligonucleotide primers:

5' PCR primer (TyrLocF2):

5'-TTTTAAGCTTGAATTCAGATCTGCCACCATGAACGGGTCTTCGGCTGC-3' (SEQ ID NO 1)

3' PCR primer (TyrLocRev):

5'-TTTTGGATCCGCGGCCGCGTCGACTCATGTCTTGAAGTGGAGCAGC-3' (SEQ ID NO 2)

The 5' primer contains the restriction sites *Hind* III, *EcoR* I and *Bgl* II, and the consensus translation enhancing sequence (GCCACC) (M. Kozak, J. Mol. Biol. (1987) 196 (4) 947-450). The 3' primer contains the restriction sites *BamH* I, *Not* I and *Sal* I. A PCR product was obtained with *Pfu* Polymerase (Stratagene) utilising the manufacturers protocol.

The following PCR conditions were used: 1 cycle of denaturation at 96°C for 2 min followed by 40 cycles of denaturation at 96°C for 1 min, annealing at 58°C for 45 s and extension at 72°C for 3 min; followed by a final extension reaction of 10 min at 72°C . The resulting PCR product was cloned into the pCR-Script (Amp SK+) vector (Stratagene) using the manufacturers protocol. Sequence analysis of a clone, confirmed the presence of a correctly edited insert. This insert was released from the pCR-Script (Amp SK+) background using *EcoR* I and *Sal* I enzymes (Pharmacia Biotech Products) and cloned by standard techniques into pEV3 digested with the same pair of enzymes (Figure 1a).

The identity of the DNA utilised for MEL cell transformation was confirmed by restriction digestion and agarose gel electrophoresis and sequencing.

Example 2

Cell culture and cell transfections

Murine erythroleukemia C88 cells (Deisseroth A Hendrick D (1978) Cell **15** 55-63) were cultured in Dulbecco's modified Eagle's medium (Gibco BRL) supplemented with 10% foetal bovine serum and 2mM glutamine at 37°C, under 10% CO2 - 90% air. "Leaky" MEL cells, C88L, which allow low level uninduced expression of globin genes in undifferentiated cells, were generated by prolonged culture of the cells (several months), prior to transfection studies.

The expression construct pEV3TyrLoc was introduced into leaky MEL-C88L cells by electroporation. Prior to transfection, 50 µg of pEV3TyrLoc were linearised at the unique *Asp* 718 site upstream of the neomycin cassette and downstream of the tyramine expression cassette. Transfection into the cell line MEL-C88L was performed by electroporation as described (Antoniou M (1991) "Induction of erythroid-specific expression in Murine Erythroleukemia (MEL) cell lines" *in* Methods in Molecular Biology Vol 7 Gene Transfer and Expression Protocols (eds Murray EJ) 421-434 The Humana Press Inc.).

After transfection, cells were diluted in culture medium to concentrations of about 10^4, 10^5 and 2×10^5 cells per ml and 1 ml aliquots were transferred to each well of a 24-well tissue culture plate (Gibco BRL-NUNC nunclon multidishes (polystyrene, radiation sterilised, with lids) 24 well plates, Cat # 143982A). Twenty four hours after the transfection, G418 was added to a concentration of 1 mg/ml in order to select for stable transfectants. Individual clones were picked, or pooled to generate populations, 7 to 10 days after the addition of selective medium.

For RNA purification and functionality studies, cells were maintained in exponential growth by passaging them every day for a period of 4 days (cells should increase from 2.10^5 cells/ml to $6\text{-}8.10^5$ cells/ml in 24 hours when in log phase). For the RNA purification, one half of the cells was induced using 2% DMSO and incubated for a further period of 4 days.

Example 3

RNA analysis

Following induction with 2% DMSO, a Northern blot was performed on RNA extracted from induced and uninduced clones and populations. Approximately 1.10^7 cells were washed with phosphate-buffered saline and resuspended in 1 ml of RNAzol B (Biogenesis). RNA was then purified according to the manufacturer's protocol. The RNA concentration was calculated from spectrophotometer readings at 260 nm.

Electrophoresis of the RNA samples (10 µg per lane) was performed through agarose gels containing 2.2 M formaldehyde in duplicate. The RNA was then transferred to a nylon membrane (Hybond-N, Amersham) in 20 x SSC. After transfer, RNA was covalently cross-linked to the membrane by short-wave ultraviolet irradiation using a U.V. Stratalinker™ 2400 (Stratagene). Each duplicate membrane was prehybridised and hybridised (Church GM & Gilbert W (1984) Proc. Natl. Acad. Sci. USA 81 1991-1995; Feinberg AP and Vogelstein

B (1983) Anal. Biochem. **132** 6-13 (Addendum: Anal. Biochem. (1984) **137** 266-267) using either ^{32}P-labeled β-globin or ^{32}P-TyrLoc-receptor probes.

There was a large increase in the production of TyrLoc mRNA after induction. Only clones that gave a strong signal on the Northern blot were used in further experiments.

Example 4

Ca^{2+} measurements

Elevations in intracellular Ca^{2+} in response to stimulation with ligands of the tyramine receptor were measured as follows. Since MEL cells loose the ability to signal on differentiation, and as receptor expression was readily detectable without induction of the MEL cells, uninduced MEL C88L cells of the invention were used.

Ca^{2+} concentration was measured by using the acetoxymethyl (AM) ester of the fluorescent indicator fura-2 (Grynkiewicz G et al. (1985) J. Biol. Chem. **260** 3440-3450). Cells were washed with NCF buffer (135 mM NaCl, 5 mM KCl, 6 mM glucose, 0.62 mM MgCl2.6H2O, 10 mM HEPES, pH 7.4 with 4 mM CaCl2) and resuspended at a concentration of 2.10^6 cells/ml in NCF buffer containing 2 μM fura-2-AM (Molecular Probes). After 1 hour incubation in the dark at 27°C, the cell suspension was subjected to centrifugation at 1200 rpm for 5 minutes, resuspended in an equal volume of NCF buffer and incubated for an additional 30 min in the dark. Aliquots were centrifuged as above and resuspended in 3 ml NCF buffer. Measurements of intracellular calcium were made fluorimetrically in a LS-50B Luminescence Spectrophotometer (Perkin-Elmer) in the presence and absence of the appropriate test chemical. Excitation wavelength alternated between 340 and 380 nm. The fluorescence intensity was monitored at an emission wavelength of 510 nm.

The different clones were tested in order to see whether there was a difference in their functional response to 10 μM tyramine (TA), an endogenous insect neurotransmitter. All of the clones tested gave a similar response (data not shown), therefore only one clone was used in further experiments. Figure 2 shows a dose-response experiment for this clone: there was a clear transient increase in Ca^{2+} after addition of TA. The lowest concentration of TA tested was 0.01 μM. A maximal response was obtained when using 1 μM TA. Untransformed MEL-C88L cells gave no response with concentrations as high as 100 μM TA.

Addition of ionomycin (2μM), a Ca^{2+}-ionophore, to the cells resulted in a large increase of the intracellular Ca^{2+} concentration, showing that the cells were loaded with suitable levels of fura-2. The Ca^{2+} dependency of the TA response was tested by using NCF buffers with different Ca^{2+} concentrations. In low Ca^{2+} buffer (no extra Ca^{2+} added to the buffer), the elevated Ca^{2+} level quickly dropped down to its initial concentration. Also, the Ca^{2+} release seen in the presence of low Ca^{2+} buffer was greater than the release seen in high Ca^{2+} buffer (4 mM Ca^{2+}) (data not shown).

These observations and results are consistent with the expectation that the initial cytoplasmic Ca^{2+} increase comes from the release of the internal stores, whilst a sustained Ca^{2+} level is achieved by an influx of Ca^{2+} from the outside of the cell. The cytosolic Ca^{2+} concentrations are probably lower in low Ca^{2+} buffer due to establishment of an equilibrium across the cell membrane. This is the reason why the fura-2 340nm/380nm fluorescence ratio increases more in low Ca^{2+} buffer, *i.e.* there was initially more free fura-2 compared to cells in high Ca^{2+} buffer.

The effect of different agonists and antagonists of the tyramine receptor was investigated. The response to octopamine (OA) was measured, since OA is a phenolamine which is structurally related to TA. Though it was used at a 50 times higher concentration, OA increased the Ca^{2+} concentration to a much lesser extent than TA, showing the specificity of the receptor for TA (Figure 3).

At concentrations below 20 μM, metaclopramide had no detectable effect on basal Ca^{2+} concentrations or on the response of the cells to TA. Chlorpromazine and mianserin clearly inhibited the Ca^{2+} increase induced by TA. Both naphazoline and tolazoline were weak antagonists (data not shown).

Yohimbine proved to be the strongest antagonist of the substances which were tested and a dose-response assay was performed (Figure 4). Yohimbine was added after 50 s at different concentrations (0.1, 1 and 2.5 μM). Subsequently, 0.5 μM and 5 μM of TA was added after 150 and 350s respectively. Yohimbine produced a concentration-dependent inhibition of the response to 0.5 μM TA, but it could not inhibit the 5 μM TA response, within the concentration range employed.

Example 5

cAMP measurements

The effect of TA on the cAMP level of the transformed and untransformed cells was investigated. MEL-C88L and MEL-TyrLoc cells were seeded in 6-well plates to a density of 8.10^5 cells/well and allowed to attach. The cell medium was removed and, in different wells, replaced with different NCF buffer solutions as follows:

(i) NCF + 1 μM tyramine (TA),

(ii) NCF + 10 μM forskolin (Fsk),

(iii) NCF + 10 μM Fsk + 1 nM to 10 μM TA.

where NCF comprised 135mM NaCl, 5mM KCl, 6mM glucose, 0.62mM $MgCl_2$, 10mM Hepes pH 7.4 4mM $CaCl_2$.

For the use of other ligands than TA, the solutions were:

(iv) NCF + 10 μM Fsk + 1 μM OA,

(v) NCF + 10 μM Fsk + 0.1 μM TA + 100 nM to 1 nM yohimbine,

(vi) NCF + 10 μM Fsk + 0.1 μM TA + 10 nM mianserin; and

(vii) NCF + 10 μM Fsk + 0.1 μM TA + 10 nM chlorpromazine.

All solutions contained 200 μM 3-iso-1-butylmethylxanthine (IBMX) in order to inhibit cAMP phosphodiesterase. In each study, triplicate wells were incubated with the same solution at 37°C for exactly 30 min.

In order to extract the cAMP, 100 % ice-cold ethanol was added to each well to a final concentration of 65 %. After 5 min incubation at room temperature, the solution was removed from the wells which were then rinsed with 65% ethanol. The eluates from the same well were then pooled together and evaporated using a speedvac. cAMP was then measured using the Scintillation Proximity Assay (Amersham), according to the manufacturer's recommended procedure. This experiment was done in triplicate.

Figure 5 shows the cAMP levels in untransformed MEL-C88L cells: addition of 1 μM TA did not result in any effect. Forskolin, with or without TA, increased the cAMP level more than 6 times.

Figure 6 shows the effect of TA on MEL-TyrLoc cells: TA significantly inhibits the forskolin-induced increase of cAMP. This response was dose-dependent; a minimal and maximal response was achieved when using 1 nM TA and 1 μM TA respectively. There was

a 4-fold decrease in cAMP production with 1 μM TA when compared to 1μM OA, therefore, indicating that TA is a much stronger inhibitor of cAMP production in these cells.

Yohimbine inhibited the effect of TA in a dose dependent manner. The antagonistic effects of mianserin and chlorpromazine were also confirmed (data not shown).

The measurements were found to be highly reproducible.

Example 6

β-galactosidase assay using dopamine clones

MEL C88L cells were transformed with the serotonin and dopamine receptors using conventional methods and from northern blot analysis, 5-6 clones were chosen for further evaluation.

Generation of pEV3D-Dop1 Expression Vector

The Dopamine Receptor cDNA from the fruit fly Drosophila melanogaster was cloned into pEV3, downstream of the human globin locus control region (LCR) between the promoter and the second intron of the β-globin gene (Figure 1a) as follows.

The coding region of the D-Dop1 cDNA was amplified by PCR from pDMdop1 (pDMdop1 contains a D-Dop1 cDNA as a partial *EcoR*Idigest from pcDNAI construct cloned into pBluescrip SK vector). (F. Gotzes et al. Receptors on Channels (1994) 2, 131-141).

The amplification was performed using the following oligonucleotide primers:

5' PCR primer (Dop D1/5 FR);

5' – TTTT AAGCTT AGATCT GCCACC ATG TAC ACA CCA ACA CCC ATTT G – 3' (SEQ IS NO 3)

3' PCR primer (Dop D1/5 RV);

5' – TTTT GC GG CC GC GTC GAC TCA AAT CGC AGACACCTGCTC – 3' (SEQ ID NO 4)

The 5' primer contains the restriction sides *Hind*III, and *Bgl*II and the consensus translation enhancing sequence (GCC ACC) (M.Kozak, 1987 supra). The 3' primer contains the restriction sites *Not* I and *Sal* I. A PCR product was obtained with *Pfu* Polymerase (Stratagene) utilising the manufacturers protocol.

The following PCR conditions were used: 1 cycle of denaturation at 96°C for 2 min followed by 35 cycles of denaturation at 96°C for 1 min, annealing at 57°C and 60°C for 45 s

and extension at 72°C for 3 min; followed by a final extension reaction of 10 min at 72°C. The resulting PCR product was cloned into the PCR-script (Amp SK+) vector (stratagene) using the manufacturers protocol. Sequence analysis of a clone, confirmed the presence of a correctly edited insert. This insert was released from the PCR-Script (AMP SK+) background using *Bgl*II and *Not*I enzymes (Pharmacia Biotech Products) and cloned into pEV3 (Figure 1a).

The identity of the DNA utilised for Mel cell transformation was confirmed by restriction digestion and agarose gel electrophoresis and sequencing.

Cell culture and cell transfection

Murine erythroleukemia C88L were cultured as described in Example 2.

The expression construct pEV3D-Dop1 and the reporter construct p3XVIP Ryg(P) See Fig 1b) were co-transformed into Mel-C88L cells by electroporation. Prior to transfection, 30µg of pEV3D-Dop1 and 30µg of p3XVIP hyg (P) were linearised respectively at the unique sites *Asp 718* and *XmnI*.

The unique *Asp718* site in the pEV3D-Dop1 vector is found upstream of the neomycin cassette and downstream of the dopamine expression cassette. The unique *XmnI* site in the p3XVIP hyg (P) vector is found in the ampicillin gene and is flanked by both the reporter expression cassette and the hygromycin cassette. Transfection into the cell line Mel-C88L was performed by electroporation as described (M. Antoniou, 1991 supra). After transfection, cells were diluted in culture medium to concentrations of about 10^4, 10^5 and $2x10^5$ cells per ml and 1ml aliquots were transferred to each well of a 24-well tissue culture plate (reference as in example 2). Twenty four hours after the transfection, G418 was added to a concentration of 1 mg/ml in order to select for stable transfectants which would contain either the pEV3 D-Dop1 expression construct on its own or with the reporter vector p3XVIP hyg (P). Individual clones were picked, or pooled to generate populations, 7 to 10 days after the addition of selective medium.

These clones and populations were visually assessed as being dividing vigorously before being passaged into media containing two selection agents: G418 and hygromycin B at respective concentrations of 1mg/ml and 0.8mg/ml. This was done in order to select only for transfectants having stably integrated both vectors: pEV3D-Dop 1 and p3XVIP. hyg(P).

For RNA purification, cells were maintained in exponential growth by passaging them every day for a period of 4 days (cells should increase from $2x10^5$ cells/ml to

6-8x10^5 cells/ml in 24 hours when in log phase). One half of the cells was then induced using 2% DMSO and incubated for a further period of 4 days. RNA analysis was carried out as described in Example 3.

β-galactosidase assays using dopamine clones

6 dopamine clones were washed in phenol red free RPMI media (GIBCO) containing 5% FCS and 1% glutamine and resuspended at concentrations of 1.25x10^7 cells/ml and 2.5x10^6 cells/ml. These cells were then transferred to 96-well microtitre plates a final concentrations of 2x10^5 cells/well and 1x10^6 cells/well. Additions of either a) Forskolin (3μM) or b) Dopamine (agonist) 10μM or c) Media were made at each of the cell concentrations. 2 replicates/clone/concentrations were set up.

After incubation for 5 hours at 37°C in an atmosphere of 10% CO_2, a chlorophenol red β-D-galactopyranoside (CPRG) solution was added. The solution comprised 11.4mg CPRG (Boehringer), 500μl 10 x Z buffer, 75μl 20% SDS, 5ml H_2O and 7μl ercaptoethanol. 10 x Z buffer consists of 0.47M $Na_2HPO_4(2H_2O)$, 0.4M $NaH_2PO_4(2H_2O)$, 0.1M KCl, 10mM $MgSO_4(7H_2O)$, adjusted to pH7.0 with NaOH solution.

Plates were then incubated overnight under the same conditions and the absorbance at 570nm determined using a spectrophotometer (MRX Microplate Reader (Dynatech Laboratories). Results for the dopamine clones at a concentration of 1x10^6 cells/well are shown in Figure 7. Clones C and 2 showed an increase in absorbance at 570nm, indicating an increase in cyclic AMP, both in the presence of forskolin and in the presence of dopamine. This signal may be useful in detecting agonists and antagonists of the dopamine receptor.

The serotonin clones showed a similar increase in cyclic AMP in the presence of forskolin. In the presence of serotonin no effect was seen but in the presence of forskolin and serotonin a decrease in forskolin stimulated cAMP was observed. This signal may be useful in detecting agonists and antagonists of the serotonin receptor.

Example 7

Generation of control MEL C88L reporter clones expressing no receptor

Cell culture and cell transfection

Murine erythroleukemia C88 "leaky" (Deisseroth A. 1978 supra) were cultured as in Example 2.

The reporter construct p3XVIP hyg(P) (Figure 1b) was transformed into Mel-C88L cells by electroporation, either on its own (Transfection 1) or in parallel with the expression vector pEV3 (Figure 1a) (Transfection 2). Prior to transfection, 30μg of pEV3 and 60μg of p3XVIP hyg (P) were linearised respectively at the unique sites *Asp 718* and *XmnI*.

The unique *Asp718* site in the pEV3 vector lies upstream of the neomycin cassette and downstream of the empty expression cassette containing the LCR enhancer. The unique *XmnI* site in the p3XVIP hyg (P) vector is found in the ampicillin gene and is flanked by both the reporter expression cassette and the hygromycin cassette. Transfections into the cell line Mel-C88L were performed by electroporation as described (M. Antoniou, 1991 supra). After each transfection, cells were diluted in culture medium to concentrations of about 10^4, 10^5 and $2x10^5$ cells per ml and 1ml aliquots were transferred to each well of a 24-well tissue culture plate (reference as in example 2). Twenty four hours after the transfection, : hygromycin B (at a concentration of 0.8mg/ml for Transfection 1) or G418 and hygromycin B (at respective concentrations of 1mg/ml and 0.8mg/ml for Transfection 2) were added in the transformations in order to select for stable transfectants which would either contain the reporter vector p3XVIP hyg (P) on its own (Transfection 1) or under the influence of the LCR enhancer from the expression vector pEV3 (Transfection 2). In Transfection 2 the double selection would select for clones containing both vectors. Individual clones were picked, or pooled to generate populations, 7 to 10 days after the addition of selective medium.

For RNA purification, cells were maintained in exponential growth by passaging them every day for a period of 4 days (cells should increase from $2x10^5$ cells/ml to $6\text{-}8x10^5$ cells/ml in 24 hours when in log phase). One half of the cells for each clone or populationwas then induced using 2% DMSO and incubated for a further period of 4 days. RNA analysis was carried out as described in Example 3.

β-galactosidase assays using p3XVIP.hyg(P) or p3XVIP.hyg(P)/pEV3 clones

12 clones and 2 populations were tested from each of Transfection 1 and 2. These clones and populations were washed in phenol red free RPMI media (GIBCO) containing 5% FCS and 1% glutamine and resuspended at an estimated concentration of between 1 and $4x10^6$ cells/ml. These cells were then transferred to 96-well microtitre plates at an estimated final concentration of between 1 to $4x10^5$ cells/well. Additions of either a) Forskolin (3.6 μM) or b) Media were made to the cells. 2 replicates/clone/concentrations were set up.

After incubation for 6 hours at 37°C in an atmosphere of 10% CO_2, a chlorophenol red β-D-galactopyranoside (CPRG) solution was added. This solution comprised 11.4mg CPRG (Boehringer), 500µl 10 x Z buffer, 75µl 20% SDS, 5ml H_2O and 7µl ercaptoethanol. 10 x Z buffer consists of 0.47M $Na_2HPO_4(2H_2O)$, 0.4M $NaH_2PO_4(2H_2O)$, 0.1M KCl, 10mM $MgSO_4(7H_2O)$, adjusted to pH7.0 with NaOH solution.

Plates were then incubated overnight under the same conditions and the absorbance at 570nm determined using a spectrophotometer (MRX Microplate Reader - Dynatech Laboratories). Results for the p3XVIP.hyg(P) and p3XVIP.hyg(P)/pEV3 clones and populations are shown in Figures 8a and 8b respectively.

The p3XVIP.hyg(P) clones and population showed no significant increase in absorbance at 570nm either in the presence or absence of forskolin: this indicates that there is no significant increase of β–galactosidase expression. This expression would have indicated an increase in cyclic AMP concentration in the cells, in turn caused by the presence of forskolin. In contrast, the p3XVIP.hyg(P)/pEV3 clones and populations showed a variety of results ranging from no significant increase in 570 nm absorbance (Clones B and F - data not shown) to near constitutive expression (Clone E and Population Pop2 - see Figure 8b) or very good inducibility of β–galactosidase expression caused by the increase of cAMP concentration in the cells in the presence of forskolin (p3XVIP.hyg(P)/pEV3 clone J and Pop1 - see Figure 8b).

This shows that both vectors are necessary for full inducibility of β–galactosidase expression by the increase of cAMP concentration in the cells. Variability in the inducibility of β–galactosidase expression between different clones is probably due to the distance, in the genome of the cell, between the LCR enhancer (pEV3; Figure 1a) and the {CRE (3xVIP) - β globin minimal promoter} which drives the expression of the LacZ gene (p3XVIP.hyg(P); Figure 1b). The optimal distance seems to have been reached in clone J (Figure 8b). This cell line can be used as a control in experiments were MEL C88L cells have been transformed with p3XVIP.hyg(P) and pEV3 expressing a heterologous protein affecting the cAMP signalling pathway.

Example 8

Generation of a parent MEL C88L {p3XVIP.hyg(P)/LCR} cell line

The experiment reported in example 7 suggested the utility in assembly of a parent cell line containing the p3XVIP.hyg(P) vector at a suitable distance from the LCR enhancer contained in pEV3. This parent cell line clone would, like p3XVIP.hyg(P)/pEV3 clone J, have easily demonstrated inducibility of β–galactosidase expression by an increase of the cAMP concentration in the cells. However, p3XVIP.hyg(P)/pEV3 clone J could not itself be utilised as parent cell line because it already contained a standard pEV3 carrying the neomycin resistance gene (and hence confering resistance to G418). The presence of this resistance gene in the parent cells hinders the transformation of this cell line with a vector allowing expression of an heterologous protein, when this protein is encoded by a gene cloned into the parent pEV3 vector. The following description details one way of obtaining a suitable parent cell line.

Generation a ΔNeomycin pEV3 Vector

In this example, the pEV3 vector was be modified to remove the neomycin gene which confers G418 resistance. Specifically the neomycin expression cassette (TK promoter - neomycin gene - Figure 1a) was removed from the pEV3 vector using *Kpn*I and *Nsp*V enzymes (Amersham Pharmacia Biotech), generating the vector ΔNeomycin pEV3.

Cell culture and cell transfection

Murine erythroleukemia C88 "leaky" (Deisseroth A, 1978 supra) were cultured as in Example 2.

The reporter construct p3XVIP hyg(P) (Figure 1b) was then co-transformed with the vector ΔNeomycin pEV3 into Mel-C88L cells by electroporation. Prior to transfection, 30μg of ΔNeomycin pEV3 and 30μg of p3XVIP hyg (P) were linearised respectively at the unique sites *Asp 718* and *Xmn*I.

The unique *Asp718* site in the pEV3 vector lies downstream of the empty expression cassette containing the LCR enhancer. The unique *Xmn*I site in p3XVIP hyg (P) vector is found in the ampicillin gene and is flanked by both the reporter expression cassette and the hygromycin cassette. Transfections into the cell line Mel-C88L were performed by electroporation as described (M. Antoniou, 1991 supra). After each transfection, cells are diluted in culture medium to concentrations of about 10^4, 10^5 and $2x10^5$ cells per ml and 1ml aliquots transferred to each well of a 24-well tissue culture plate (reference as in example 2).

Twenty four hours after the transfection, hygromycin B (at a concentration of 0.8mg/ml) was added in the transformation in order to select for stable transfectants which would either contain the reporter vector p3XVIP hyg (P) on its own or under the influence of the ΔNeomycin pEV3 vector's LCR enhancer (it was not be possible to select for the double transformants directly as the ΔNeomycin pEV3 vector does not contain any markers allowing selection in mammalian cells). Individual clones were picked, or pooled to generate populations, 7 to 10 days after the addition of selective media.

For RNA purification, cells were maintained in exponential growth by passaging every day for a period of 4 days (cells grow from $2x10^5$ cells/ml to $6\text{-}8x10^5$ cells/ml in 24 hours when in log phase). One half of the cells for each clone or population was then induced using 2% DMSO and incubated for a further period of 4 days. RNA analysis was carried out as described in Example 3.

β-galactosidase assays using p3XVIP.hyg(P) or p3XVIP.hyg(P)/ ΔNeomycin pEV3 clones

50 clones and 2 populations were tested for inducibility of β−galactosidase expression by the increase of cAMP concentration in the cells. The clones and populations were washed in phenol red free RPMI media (GIBCO) containing 5% FCS and 1% glutamine and then resuspended at a concentration of between 1 and $4x10^6$ cells/ml. Cell suspensions were then transferred to 96-well microtitre plates at a final concentration of between 1 to $4x10^5$ cells/well. Additions of either a) Forskolin (3 to 3.6 μM) or b) Media were made to the cells. 2 replicates/clone/concentrations were set up.

After incubation for 5 to 6 hours at 37°C in an atmosphere of 10% CO_2, a chlorophenol red β-D-galactopyranoside (CPRG) solution was added. This solution comprised 11.4mg CPRG (Boehringer), 500μl 10 x Z buffer, 75μl 20% SDS, 5ml H_2O and 7μl mercaptoethanol. 10 x Z buffer consists of 0.47M $Na_2HPO_4(2H_2O)$, 0.4M $NaH_2PO_4(2H_2O)$, 0.1M KCl, 10mM $MgSO_4(7H_2O)$, adjusted to pH7.0 with NaOH solution.

Plates were incubated overnight under the same conditions and the absorbance at 570nm was determined using a spectrophotometer (MRX Microplate Reader - Dynatech Laboratories). The results from this experiment were similar to those obtained in example 7, with a mixture of cells responding either like Transfection 1 or 2 clones. This is due to the fact that in this transfection, there were a mixture of clones produced and containing either p3XVIP hyg(P) vector on its own or co-integrated with ΔNeomycin pEV3 vector, because of the simple selection with hygromycin. A parent MEL C88L (p3XVIP.hyg(P)/LCR) cell line

was then selected on the basis of criteria including optimal inducibility of β–galactosidase expression by the increase of cAMP concentration in the cells, as for p3XVIP.hyg(P)/pEV3 clone J.

Other modifications of the present invention will be apparent to those skilled in the art without departing from the scope of the invention.

CLAIMS

1. The use of an erythroid cell which is substantially undifferentiated, but which is capable of expressing a heterologous protein under the control of a globin promoter thereof, in an assay in which said protein interacts with an endogenous signalling cascade of said cell and said interaction is detected.

2. The use according to claim 1 wherein said erythroid cell is a murine erythroleukaemia (MEL) cell, rat erythroleukaemia cell (REL) or a human erythroleukaemia cell (HEL).

3. The use according to claim 2 wherein the erythoid cell is a murine erythroleukaemia cell.

4. The use according to any one of claims 1 to 3 wherein the said globin promoter is the β-globin promoter.

5. An erythoid cell which is substantially undifferentiated but which is capable of expressing proteins under the control of a globin promoter thereof at levels which allow use in accordance with any one of claims 1 to 4.

6. An erythoid cell according to claim 5 which comprises a cell as deposited at the European Collection of Cell Cultures under Accession number 99012801.

7. A method of producing an erythroid cell according to claim 5 which method comprises maintaining growing uninduced erythroid cells in culture for a sufficient period of time and isolating a subclone which expresses protein under the control of a globin promoter.

8. A method for determining the interaction between a receptor protein and a potential agonist or antagonist therefor, said method comprising incubating a cell as defined above which has been transformed so that it expresses said receptor protein as a G-protein coupled receptor, either

(I) in (a) the presence and (b) the absence of said potential agonist; and/or

(II) in the presence of a known agonist and (a) the presence or (b) the absence of said potential antagonist; and

monitoring and comparing G-protein coupled receptor induced signals in cells of (Ia) and (Ib) and/or (IIa) and (IIb).

9. A method according to claim 8 wherein the G-protein coupled receptor induced signal is monitored by measuring the calcium ion content of the cells.

10. A method according to claim 9 wherein the calcium levels are measured by means of a fluorescent indicator.

11. A method according to claim 8 wherein the G-protein coupled receptor induced signal results in a change in the cyclic AMP (cAMP) levels within the cell, and the G-protein induced signal is monitored by measuring the cyclic AMP content of the cells.

12. A method according to claim 11 wherein the cells are transformed with a reporter gene, expressed of which is regulated by a G-protein coupled receptor induced signalling cascade, and the G-protein coupled receptor induced signal is monitored by detecting the product of the reporter gene.

13. A method according to claim 12 wherein the reporter gene is β-GAL.

14. A method according to any one of claims 8 to 13 wherein the G-protein coupled receptor induced signal results in a decrease in the level of the measured cellular component, and tests (I) and (II) are carried out in the presence of a chemical which contributes to an increased level of said cellular component.

15. A method according to claim 14 wherein the measured cellular component is cAMP and the said chemical is forskolin.

16. A method according to any one of claims 7 to 15 wherein the receptor is an insect receptor.

17. A method according to claim 16 wherein the insect receptor is a tyramine, a serotonin, a dopamine, an octopamine or a muscarinic -acetylcholine receptor.

18. A method according to any one of claims 8 to 17 wherein the cells are subsequently induced to differentiate, and used in a ligand binding assay.

19. An assay for detecting binding between a protein and a potential binding partner therefore, said method comprising (a) transforming a cell according to claim 5 so that the protein is expressed under the control of a globin promoter, (b) detecting binding between said potential binding partner and the said protein on a membrane of the cell.

20. An assay according to claim 19 where the cells are induced after step (a) and prior to step (b), so as to obtain high levels of protein expression from fully differentiated cells.

21. An assay according to claim 19 or claim 20 wherein step (b) may be effected on isolated membranes extracted from lysed cells.

22. A vector comprising a sequence which encodes a non-mammalian protein receptor under the control of a globin promoter.

23. A vector according to claim 22 wherein the globin promoter is under the control of the human globin locus control region.

24. A vector according to claim 22 or claim 23 wherein the non-mammalian protein receptor is an insect receptor.

25. A vector according to claim 24 wherein the insect receptor is the locust tyramine receptor.

26. A cell according to claim 5 or claim 6 which is transformed with a vector according to any one of claims 22 to 25.

27. A cell according to claim 5 which has been transformed such that it contains a globin promoter associated with a cloning site and/or a reporter cassette containing a reporter gene, such as the β–galactosidase gene, under the control of a response element susceptible to modulation by a signalling cascade used in an assay.

28. A cell according to claim 26 which further comprises an enhancer, able to increase expression of a gene placed under the control of said globin promoter and/or is at an optimal distance of said reporter cassette such that the expression is dependent on the concentration of a particular downstream component in the signalling cascade.

29. A cell according to claim 28 wherein the enhancer is the LCR enhancer.

FIG 1a

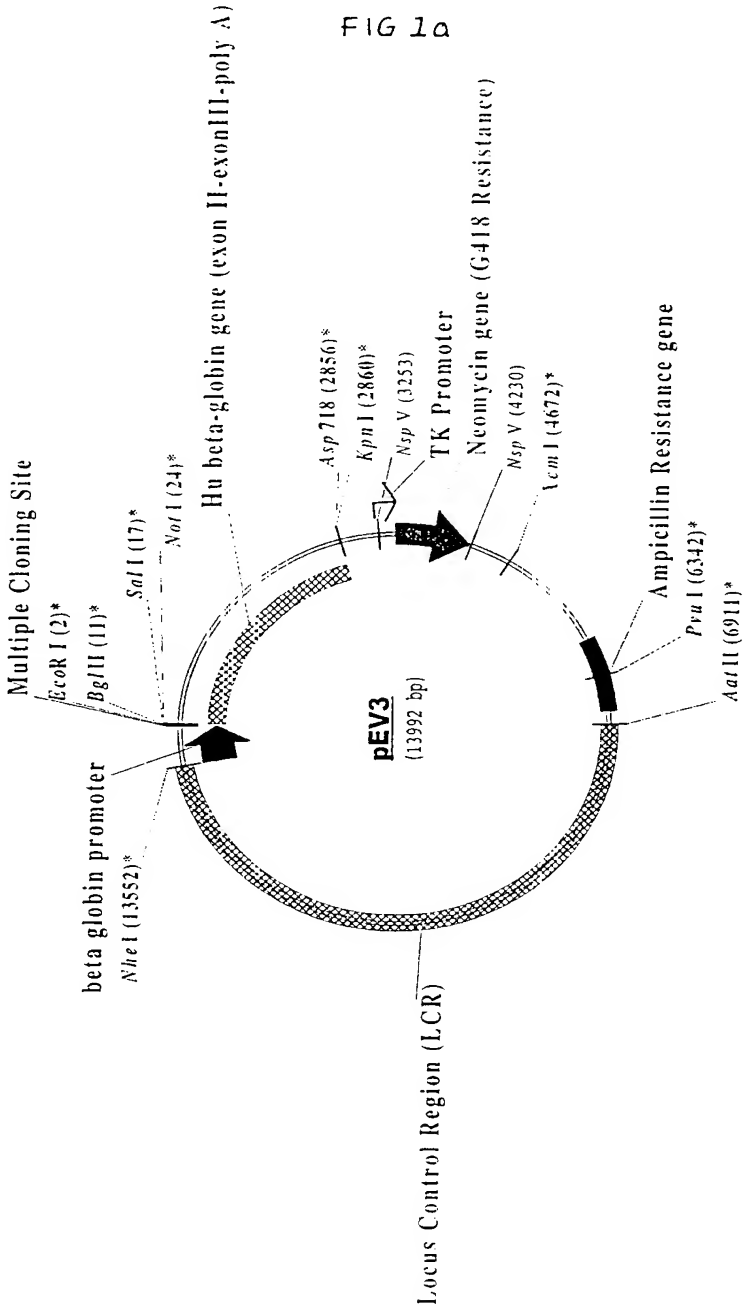

Multiple Cloning Site
EcoR I (2)*
BglII (11)*
Sal I (17)*
Not I (24)*
Hu beta-globin gene (exon II-exonIII-poly A)
Asp 718 (2856)*
Kpn I (2860)*
Nsp V (3253)
TK Promoter
Neomycin gene (G418 Resistance)
Nsp V (4230)
Xcm I (4672)*
Ampicillin Resistance gene
Pvu I (6342)*
Aat II (6911)*
pEV3
(13992 bp)
beta globin promoter
Nhe I (13552)*
Locus Control Region (LCR)

FIG 1b

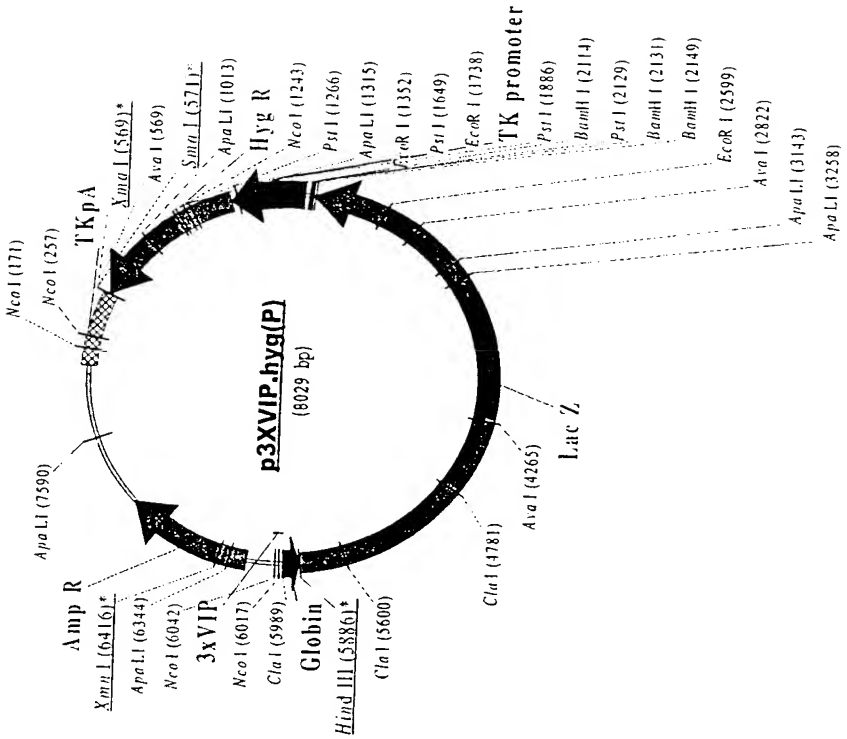

p3XVIP.hyg(P)
(8029 bp)
TKpA
Hyg R
TK promoter
Lac Z
Amp R
3xVIP
Globin
Nco I (171)
Nco I (257)
Xma I (569)*
Ava I (569)
Sma I (571)*
Apa LI (1013)
Nco I (1243)
Pst I (1266)
Apa LI (1315)
EcoR I (1352)
Pst I (1649)
EcoR I (1738)
Pst I (1886)
BamH I (2114)
Pst I (2129)
BamH I (2131)
BamH I (2149)
EcoR I (2599)
Ava I (2822)
Apa LI (3143)
Apa LI (3258)
Ava I (4265)
Cla I (4781)
Cla I (5600)
Hind III (5886)*
Nco I (6017)
Cla I (5989)
Apa LI (6344)
Nco I (6042)
Xmn I (6416)*
Apa LI (7590)

FIG 2

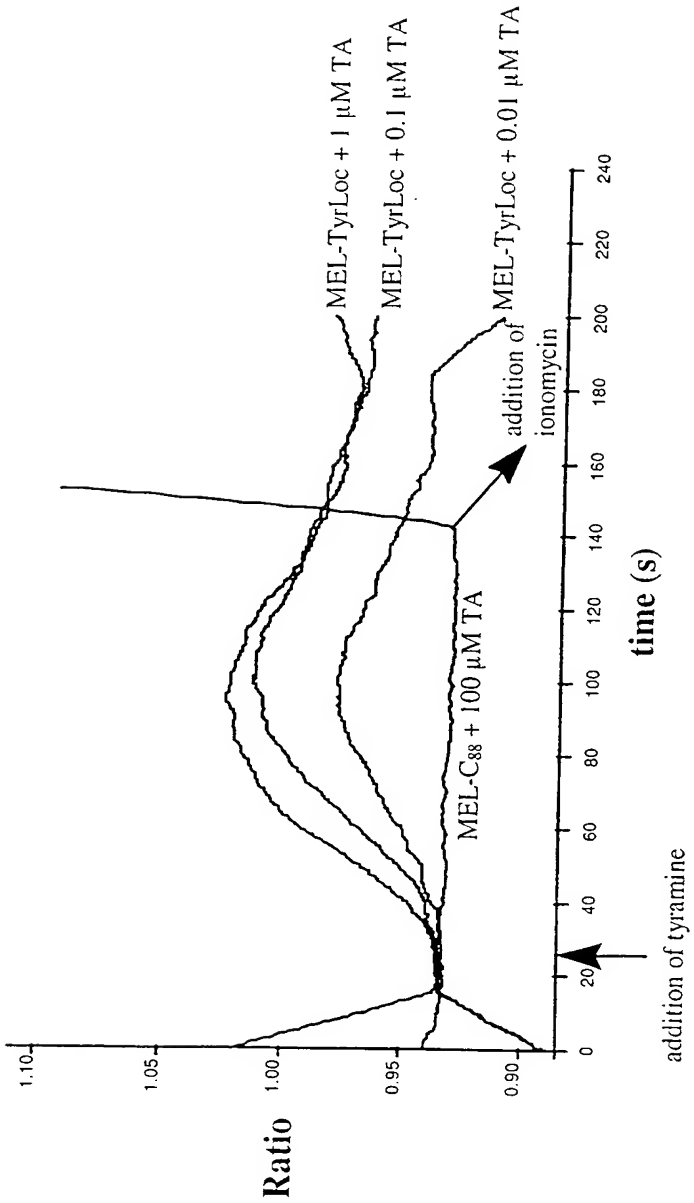

MEL-TyrLoc + 1 µM TA
MEL-TyrLoc + 0.1 µM TA
MEL-TyrLoc + 0.01 µM TA
MEL-C88 + 100 µM TA
addition of ionomycin
addition of tyramine
Ratio
time (s)
1.10
1.05
1.00
0.95
0.90
0
20
40
60
80
100
120
140
160
180
200
220
240

FIG 3

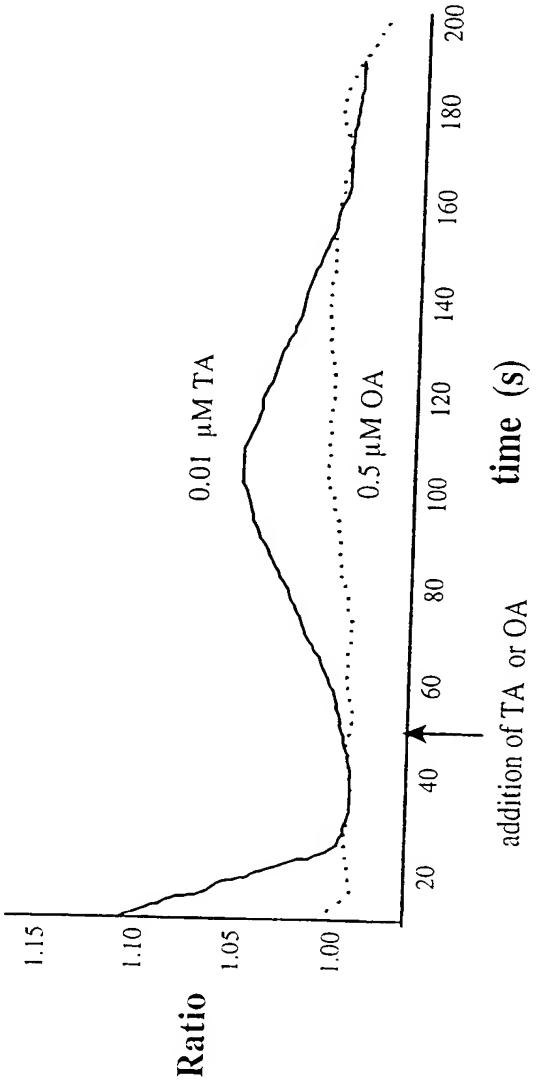

Ratio
1.15
1.10
1.05
1.00
0.01 µM TA
0.5 µM OA
addition of TA or OA
time (s)
20
40
60
80
100
120
140
160
180
200

FIG 4

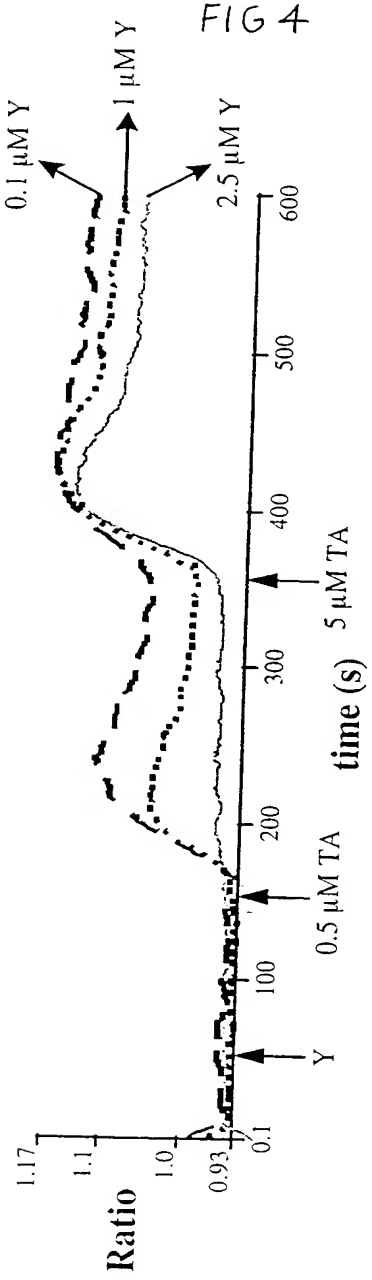

0.1 µM Y
1 µM Y
2.5 µM Y
Y
0.5 µM TA
5 µM TA
time (s)
Ratio
1.17
1.1
1.0
0.93
0.1
100
200
300
400
500
600

FIG 5

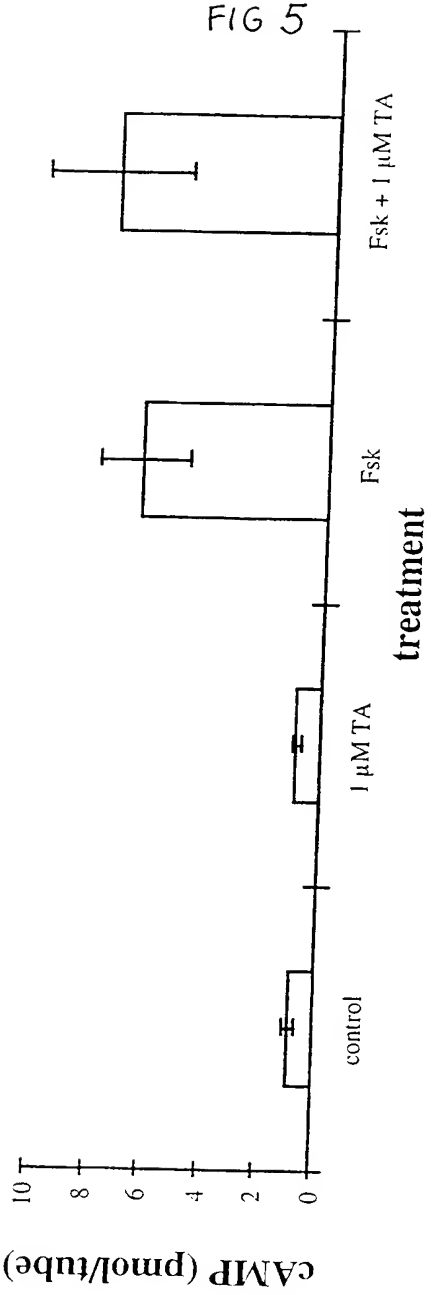

cAMP (pmol/tube)
0
2
4
6
8
10
control
1 μM TA
Fsk
Fsk + 1 μM TA
treatment

FIG 6

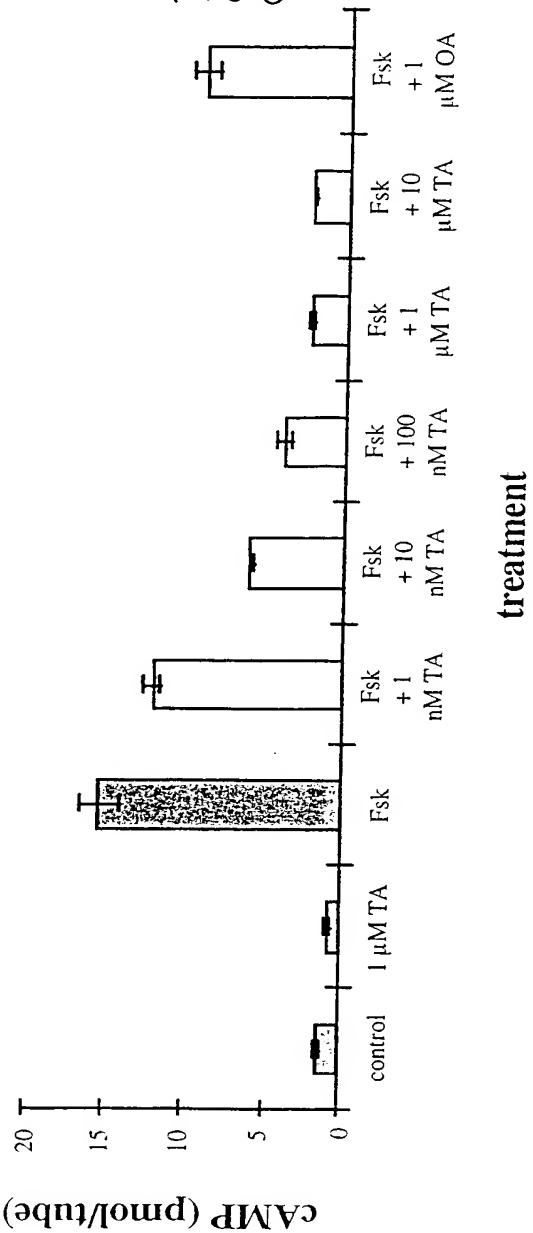

cAMP (pmol/tube)
0
5
10
15
20
control
1 µM TA
Fsk
Fsk + 1 nM TA
Fsk + 10 nM TA
Fsk + 100 nM TA
Fsk + 1 µM TA
Fsk + 10 µM TA
Fsk + 1 µM OA
treatment

FIG 7

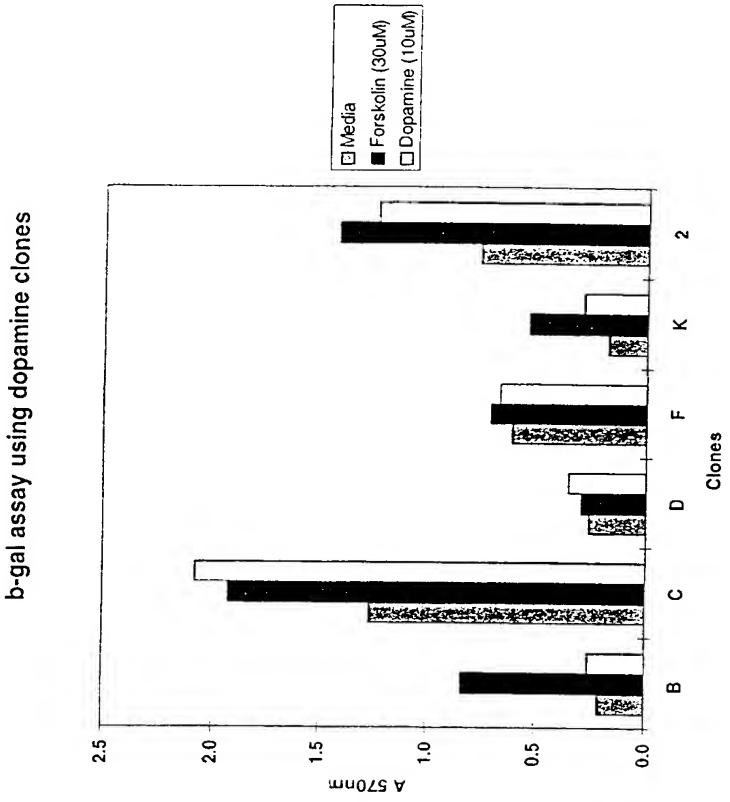

b-gal assay using dopamine clones
Media
Forskolin (30uM)
Dopamine (10uM)
A 570nm
2.5
2.0
1.5
1.0
0.5
0.0
B
C
D
F
K
2
Clones

FIG 8a

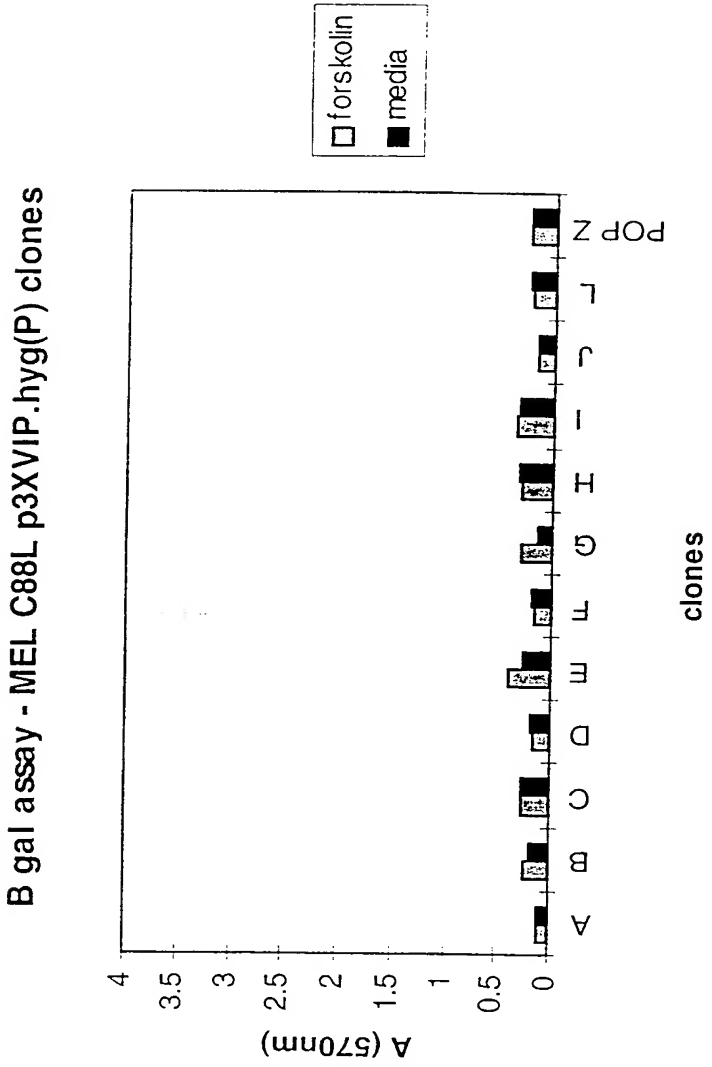
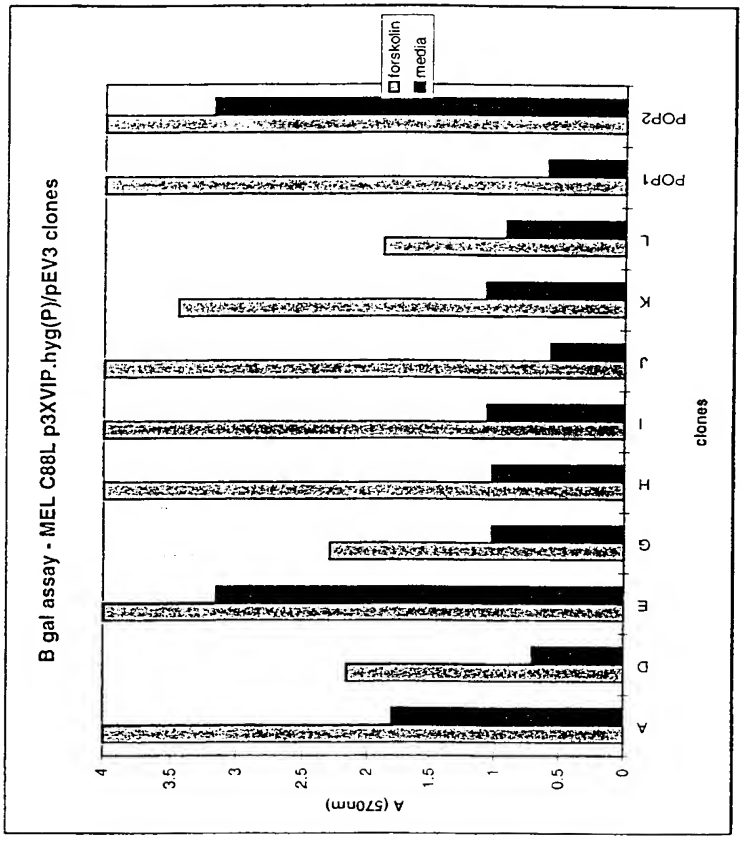

B gal assay - MEL C88L p3XVIP.hyg(P) clones
forskolin
media
A (570nm)
0
0.5
1
1.5
2
2.5
3
3.5
4
A
B
C
D
E
F
G
H
I
J
L
POP Z
clones

FIG 8b


B gal assay - MEL C88L p3XVIP.hyg(P)/pEV3 clones
forskolin
media
A (570nm)
0
0.5
1
1.5
2
2.5
3
3.5
4
A
D
E
G
H
I
J
K
L
POP1
POP2
clones

SEQUENCE LISTING

<110> ZENECA LIMITED

<120> CELLS AND ASSAY

<130> PPD50368/WO

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<151> 1999-05-07

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<170> PatentIn Ver. 2.0

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<213> Artificial Sequence

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<210> 3
<211> 45
<212> DNA
<213> Artificial Sequence

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<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence:PCR Primer DopD1/5RV

<400> 4
ttttgcggcc gcgtcgactc aaatcgcaga cacctgctc 39

PATENT COOPERATION TREATY

From the
INTERNATIONAL PRELIMINARY EXAMINING AUTHORITY

To:

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Jealott's Hill International
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Bracknell, Berkshire RG42 6YA
GRANDE BRETAGNE

PCT

NOTIFICATION OF TRANSMITTAL OF THE INTERNATIONAL PRELIMINARY EXAMINATION REPORT

(PCT Rule 71.1)

	Date of mailing *(day/month/year)* 13.08.2001
Applicant's or agent's file reference PPD 50368/WO	**IMPORTANT NOTIFICATION**

International application No.	International filing date *(day/month/year)*	Priority date *(day/month/year)*
PCT/GB00/01702	04/05/2000	07/05/1999

Applicant
SYNGENTA LIMITED et al.

1. The applicant is hereby notified that this International Preliminary Examining Authority transmits herewith the international preliminary examination report and its annexes, if any, established on the international application.

2. A copy of the report and its annexes, if any, is being transmitted to the International Bureau for communication to all the elected Offices.

3. Where required by any of the elected Offices, the International Bureau will prepare an English translation of the report (but not of any annexes) and will transmit such translation to those Offices.

4. **REMINDER**

The applicant must enter the national phase before each elected Office by performing certain acts (filing translations and paying national fees) within 30 months from the priority date (or later in some Offices) (Article 39(1)) (see also the reminder sent by the International Bureau with Form PCT/IB/301).

Where a translation of the international application must be furnished to an elected Office, that translation must contain a translation of any annexes to the international preliminary examination report. It is the applicant's responsibility to prepare and furnish such translation directly to each elected Office concerned.

For further details on the applicable time limits and requirements of the elected Offices, see Volume II of the PCT Applicant's Guide.

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Form PCT/IPEA/416 (July 1992)

PATENT COOPERATION TREATY

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INTERNATIONAL PRELIMINARY EXAMINATION REPORT

(PCT Article 36 and Rule 70)

Applicant's or agent's file reference	FOR FURTHER ACTION	See Notification of Transmittal of International Preliminary Examination Report (Form PCT/IPEA/416)
PPD 50368/WO		
International application No.	International filing date *(day/month/year)*	Priority date *(day/month/year)*
PCT/GB00/01702	04/05/2000	07/05/1999
International Patent Classification (IPC) or national classification and IPC C12N5/16		
Applicant SYNGENTA LIMITED et al.		

1. This international preliminary examination report has been prepared by this International Preliminary Examining Authority and is transmitted to the applicant according to Article 36.

2. This REPORT consists of a total of 6 sheets, including this cover sheet.

   ☐ This report is also accompanied by ANNEXES, i.e. sheets of the description, claims and/or drawings which have been amended and are the basis for this report and/or sheets containing rectifications made before this Authority (see Rule 70.16 and Section 607 of the Administrative Instructions under the PCT).

   These annexes consist of a total of sheets.

3. This report contains indications relating to the following items:

   - I [X] Basis of the report
   - II [ ] Priority
   - III [ ] Non-establishment of opinion with regard to novelty, inventive step and industrial applicability
   - IV [ ] Lack of unity of invention
   - V [X] Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations suporting such statement
   - VI [ ] Certain documents cited
   - VII [ ] Certain defects in the international application
   - VIII [X] Certain observations on the international application

Date of submission of the demand	Date of completion of this report
10/10/2000	13.08.2001
Name and mailing address of the international preliminary examining authority: European Patent Office D-80298 Munich Tel. +49 89 2399 - 0 Tx: 523656 epmu d Fax: +49 89 2399 - 4465	Authorized officer Ury, A Telephone No. +49 89 2399 8411

Form PCT/IPEA/409 (cover sheet) (January 1994)

INTERNATIONAL PRELIMINARY EXAMINATION REPORT

International application No. PCT/GB00/01702

I. Basis of the report

1. With regard to the **elements** of the international application *(Replacement sheets which have been furnished to the receiving Office in response to an invitation under Article 14 are referred to in this report as "originally filed" and are not annexed to this report since they do not contain amendments (Rules 70.16 and 70.17))*:
Description, pages:

1-25 as originally filed

Claims, No.:

1-29 as originally filed

Drawings, sheets:

1/10-10/10 as originally filed

2. With regard to the **language**, all the elements marked above were available or furnished to this Authority in the language in which the international application was filed, unless otherwise indicated under this item.

These elements were available or furnished to this Authority in the following language: , which is:

- ☐ the language of a translation furnished for the purposes of the international search (under Rule 23.1(b)).
- ☐ the language of publication of the international application (under Rule 48.3(b)).
- ☐ the language of a translation furnished for the purposes of international preliminary examination (under Rule 55.2 and/or 55.3).

3. With regard to any **nucleotide and/or amino acid sequence** disclosed in the international application, the international preliminary examination was carried out on the basis of the sequence listing:

- ☐ contained in the international application in written form.
- ☐ filed together with the international application in computer readable form.
- ☐ furnished subsequently to this Authority in written form.
- ☐ furnished subsequently to this Authority in computer readable form.
- ☐ The statement that the subsequently furnished written sequence listing does not go beyond the disclosure in the international application as filed has been furnished.
- ☐ The statement that the information recorded in computer readable form is identical to the written sequence listing has been furnished.

4. The amendments have resulted in the cancellation of:

- ☐ the description, pages:
- ☐ the claims, Nos.:

☐ the drawings, sheets:

5. ☐ This report has been established as if (some of) the amendments had not been made, since they have been considered to go beyond the disclosure as filed (Rule 70.2(c)):

(Any replacement sheet containing such amendments must be referred to under item 1 and annexed to this report.)

6. Additional observations, if necessary:

V. Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement

1. Statement

Novelty (N)	Yes:	Claims	1-4, 6, 8-17, 22-29
	No:	Claims	5, 7, 18-21
Inventive step (IS)	Yes:	Claims	1-4, 8-17
	No:	Claims	5-7, 18-29
Industrial applicability (IA)	Yes:	Claims	1-29
	No:	Claims	

2. Citations and explanations
see separate sheet

VIII. Certain observations on the international application

The following observations on the clarity of the claims, description, and drawings or on the question whether the claims are fully supported by the description, are made:
see separate sheet

Item V.

Reference is made to the following documents:

D1: Garcia-Alonso M. et al.:' STABLE FUNCTIONAL EXPRESSION OF THE NEURONAL NICOTINIC ACETYLCHOLINE RECEPTOR alpha3beta4 IN MEL (MURINE ERYTHROLEUKEMIA) CELLS: A NOVEL EXPRESSION SYSTEM FOR LIGAND GATED ION CHANNELS' SOCIETY FOR NEUROSCIENCE ABSTRACTS vol. 22,1996, page 1526 XP000961688

D2: GB-A-2 251 622 (ICI PLC) 15 July 1992 (1992-07-15) cited in the application

D3: NEEDHAM M ET AL: 'LCR/MEL: A VERSATILE SYSTEM FOR HIGH-LEVEL EXPRESSION OF HETEROLOGOUS PROTEINS IN ERYTHROID CELLS' NUCLEIC ACIDS RESEARCH, vol. 20, no. 5, 11 March 1992 (1992-03-11), pages 997-1003, XP000602190 ISSN: 0305-1048 cited in the application

I) Document D1 discloses an erythroid cell (MEL cell) containing and expressing a heterologous protein (the neuronal nicotinic acetylcholine receptor alpha3β4) under the control of a globin promoter and the LCR (MEL/LCR system). Said MEL cells were induced to differentiate with DMSO.
D1 further discloses that said MEL/LCR system affords advantages for the detection of functional activity of receptors and ion channels.
In other words, D1 discloses or at least suggests the method according to present claims 18-21 (Article 33.2 and 3 PCT).

II) The difference between the vectors disclosed in D3 (page 998) and the vector according to present claims 22-25 is that the latest comprises a sequence which encodes a non-mammalian protein receptor.
The difference between the vector disclosed in D1 (second part of the document) and the vector according to present claims 22-25 is that the latest comprises a sequence which encodes a protein receptor which is non-mammalian.
In view of the advantages of the MEL/LCR system for reproducible, high-level expression and stable expression of heterologous proteins of interest (see D1 and D3), the skilled person would have used said system for the production of e.g. non-mammalian protein receptor. The substitution of the proteins according to D1

and D3 with a non-mammalian protein receptor in the MEL/LCR system merely consists in an obvious alternative which does not involve an inventive step. Therefore, the vector (intermediate product) according to claims 22-25 does not fulfil the requirements of Article 33.3 PCT.

The undifferentiated erythroid cell according to claim 26 is an intermediate cell obtained in the MEL/LCR system before induction of differenciation has started. Said cell is also not inventive since it is compulsorily obtained in the procedure.

III) The undifferentiated erythroid cell according to claim 5 is an intermediate cell obtained in the MEL/LCR system disclosed in D1 and D3 before induction of differenciation has started. Thus, claim 5 lacks novelty (Article 33.2 PCT). The method according to claim 7 is also disclosed in D3 (pages 998-999).

Dependent claims 6, 27-29 do not seem to contain any feature which, in combination with the features of the claims to which they refer, meet the requirements of the PCT in respect of inventive step.

IV) D2, figs.13 and 14 (see NI = non-induced) also destroy the novelty of present claims 5 and 6.

V) In the conventional MEL/LCR system, induction of differentiation frequently leads to the loss of functionality of the signalling cascades linked to G-protein coupled receptors. Thus recombinant MEL cell cannot be used in functional assays with G-protein coupled receptors. The present invention is based on the fact that undifferentiated erythroid cells (MEL cells for instance) can be used in functional assays with G-protein coupled receptors (Ca++, IP3, or cAMP assays) since the signalling cascades linked to G-protein coupled receptors are still functional. This was neither disclosed nor suggested in the cited prior art.
Thus, it would seem that the subject-matter of claims 1-4 and 8-17 fulfil the requirements of Article 33.2 and 3 PCT.

Item VIII.

1) It is clear from the description that the "heterologous protein" (claim 1) and the "receptor protein" (claim 8) should be defined as being a "G-protein coupled receptor". This feature is essential to the definition of the invention.
Since independent claims 1 and 8 do not contain this feature they do not meet the requirement following from Article 6 PCT taken in combination with Rule 6.3(a) PCT that any independent claim must contain all the technical features essential to the definition of the invention.

2) The term "substantially" used in claims 1, 5 is vague and unclear and leaves the reader in doubt as to the meaning of the technical features to which it refers, thereby rendering the definition of the subject-matter of said claims unclear (Article 6 PCT).

3) The expressions "thereof" (claims 1, 5) and "a cell as defined above" (claim 8) are unclear (Article 6 PCT).